Charles University in Prague
Faculty of Science

Modulation of memory using enzymatic digestion of perineuronal nets in perirhinal cortex

Modulace paměťových stop pomocí enzymatického štěpení perineurálních sítí v oblasti perirhinální kůry

Bc. Natálie Procházková

Praha 2019

Školitel: doc. RNDr. Pavla Jendelová, Ph.D.
Konzultant: RNDr. Jiří Růžička, Ph.D.

Prohlášení:

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, dne

Podpis

Poděkování

Ráda bych poděkovala mé školitelce doc. RNDr. Pavle Jendelové, Ph.D. za odborné vedení mé diplomové práce, cenné rady a vstřícný přístup. Dále bych chtěla poděkovat RNDr. Jiřímu Růžičkovi, Ph.D. za odborné konzultace v ohledu experimentálních metod a přínosné připomínky při vypracování mé diplomové práce. Na závěr mé velké díky patří mé rodině a blízkým, kterým vděčím za podporu v průběhu celého studia.

Abstract

In the adult brain, neuronal plasticity is regulated by a specialized structure of extracellular matrix, the perineuronal nets (PNNs), which restrict synaptic plasticity by binding molecules of inhibitory nature and posing as a physical barrier to alterations in neuronal connectivity. This effect is abolished by removal of PNNs by the enzyme chondroitinase ABC (chABC), which enables to reopen critical period window and leads to memory improvement. Here, we utilized chABC and a novel approach in removing the PNNs in perirhinal cortex, using the enzyme hyaluronidase (Hyase), to assess differences in the use of these enzymes in object recognition (OR) memory improvement and alterations in the structure of neuronal network of wild type mice. Our findings suggest that Hyase may be a more convenient tool to PNN removal than chABC, as Hyase surpasses chABC in promoting the OR memory, influence larger portion of neuronal network by affecting both inhibitory and excitatory neurons, and provides extended temporal window for experimental modulation of activity-dependent synaptic plasticity.

Key words: perineuronal nets, synaptic plasticity, chondroitinase ABC, hyaluronidase, perirhinal cortex, memory

Abstrakt:

Plasticita neuronové sítě v dospělém mozku je regulována specializovanou strukturou extracelulární matrix – perineurálními sítěmi (PNS), které jsou zodpovědné za restrikci synaptické plasticity vazbou inhibičních molekul a zabraňují změnám v neuronální konektivitě. Odstraněním PNS pomocí enzymu chondroitinázy ABC lze tomuto efektu předejít a znovu tak otevřít období kritické periody, což vede ke zlepšení paměti umožňující rozeznávat nové předměty (object recognition – OR). V této studii jsme k odstranění PNS použili chABC a enzym dosud nepoužívaný k odstranění PNS v perirhinálním kortexu, hyaluronidázu (Hyázu), abychom zjistili rozdíly v jejich efektu na OR paměť a na následné změny ve struktuře nervové sítě wild type myší. Naše výsledky ukazují, že Hyáza má potenciál sloužit jako vhodnější prostředek k odstranění PNS, jelikož překonává chABC ve zvýšení OR paměti, ovlivňuje rozsáhlejší oblast nervové sítě díky působení na inhibiční i excitační neurony, a umožňuje prodloužit časové období zvýšené synaptické plasticity.

Klíčová slova: perineurální sítě, synaptická plasticita, chondroitináza ABC, hyaluronidáza, perirhinální kortex, paměť

Table of Contents

1. List of abbreviations

AAV	adeno-associated virus
Acan	Aggrecan
AD	Alzheimer's disease
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AMPAR	AMPA receptors
Bral2	Brain link protein 2
C6ST1	chondroitin 6-sulfotransferase-1
chABC	Chondroitinase ABC
CNS	central nervous system
Crtl1	Cartilage link protein Crtl1
CSGalNAcT- 1	chondroitin sulphate N-acetylgalactosaminyltransferase-1
CSPG	chondroitin sulphate proteoglycan
ECM	extracellular matrix
GABA	gamma-aminobutyric acid
GAD 65/67	glutamate acid decarboxylase (GAD67)
GalNAc	N-acetylgalactosamine
GlcA	glucuronic acid
HA	hyaluronic acid
HAPLN	hyaluronan- and proteoglycan-binding link protein gene family
HAS	hyaluronan synthase
Hyase	Hyaluronidase
IHC	immunohistochemistry
LTD	long-term depression
LTP	long-term potentiation
NG2	Neuroglia 2
OR	object recognition
ORT	object recognition task
Otx2	Orthodenticle homeobox 2
PNN	perineuronal net
PPR	paired-pulse ratio

PSD95	Postsynaptic density 95
PV	parvalbumine
ROS	reactive oxygen species
Sema3A	Semaphorin 3A
SNAP25	Synaptosomal nerve-associated protein 25
Tn-R	Tenascin R
VAMP2	Vesicle-associated membrane protein 2
VGAT	Vesicular GABA transporter
VGLUT	Vesicular glutamate transporter
wt	wild type

2. Introduction

Perineuronal nets (PNNs) are a specialized structure of condensed extracellular matrix (ECM) present in the central nervous system (CNS), where they ensheath the soma and proximal dendrites of several neuronal populations, preferentially the gamma-aminobutyric acid (GABA)-ergic fast-spiking parvalbumine-positive (PV+) interneurons (Celio and Blumcke, 1994). The formation of PNNs, which occurs late in the postnatal development (Carulli *et al.*, 2010), marks the closure of the critical period, substantial for brain network maturation (Pizzorusso, 2002). In the adult organism, their principal function is to restrict synaptic plasticity, which is achieved by their inhibitory properties on synapse formation and axonal sprouting. Moreover, these features of PNNs are essential in regulating the processes of learning and memory acquisition (Duncan *et al.,* 2019). PNNs also support the high activity of fast-spiking PV+ interneurons by providing suitable microenvironment and buffering cations released during action potentials and protecting them from oxidative stress (Morawski *et al.*, 2004; Cabungcal *et al.*, 2013). PNNs have been known since 1889, when they were first observed by Camillo Golgi; however, their structure and properties were elucidated not earlier than one hundred years later in the 1990-2000's. Since then, there have been numerous studies providing data of the significant role of PNNs in development and function in healthy, aging or pathological CNS. Blocking the formation of the PNNs prolongs the critical period (Carulli *et al.*, 2010) and removal of PNNs in the adult organism reopens the critical period window (Pizzorusso, 2002). That will shift the CNS in a juvenile-like state with high plasticity, prone to formation of new synapses and remodeling neuronal connectivity network (Fawcett, 2015). Removal of PNNs enables to restore ocular dominance (Pizzorusso, 2002), increase axonal terminals sprouting (Kwok *et al.*, 2011) and enhance memory retention in mice (Romberg *et al.*, 2013). PNNs removal is mostly done by local administration of bacterial enzyme, the Chondroitinase ABC (chABC). We compare the use of chABC with another enzyme used to digest PNN, Hyaluronidase (Hyase), and assess their effect on learning and memory.

3. Current state of knowledge

3.1 What are the PNNs

The major components of brain extracellular space are diffused matrix, condensed matrix and basement membrane, which forms a barrier separating parenchymal tissue and endothelia (Lau *et al.*, 2013). While most of the extracellular space is occupied by the diffused matrix, a subset of neurons forms the condensed component of the ECM, called perineuronal nets.

PNNs form a lattice-like layer of molecules that ensheath synapses of the soma, proximal dendrites and axonal initial segment of subpopulation of neurons (Hockfield and McKay, 1983; Celio and Blumcke, 1994), leaving holes for axonal boutons (Hockfield and McKay, 1983; Brückner *et al.,* 1993). PNNs can be found around several types of interneurons, especially the PV+ fast-spiking GABA-ergic neurons. In a small extent, they are also present around some types of excitatory neurons, such as pyramidal cells in the cerebral cortex (Brückner *et al.*, 1993; Brückner *et al.,* 2004; Morawski *et al.*, 2004). PNNs of excitatory and inhibitory neurons differ significantly in their composition, and excitatory neurons form notably more delicate PNNs with lesser amounts of proteoglycans (Hausen *et al.,* 1996; Wegner *et al.,* 2003). PNNs are present in many areas of both brain and spine of various species: they have been found and studied in mice, rats, cats and songbirds and they are present also in the human CNS (Seeger *et al.*, 1994; Morawski *et al.*, 2004).

3.2 PNN structure

The structure of PNNs (Fig. 1) contain various components, all of which play a specific role in the whole complex. The backbone of PNNs is composed of hyaluronan (hyaluronic acid - HA), which is comprised of alternating disaccharide units of *N*-acetylglucosamine and glucuronic acid (Meyer *et al.*, 1951). These form non-sulfated, long polymers, branching and binding together into a mesh-like structure, which directly determines the architecture of PNNs (Spicer *et al.,* 2003; McRae and Porter, 2012). HA is a component of PNNs, which is crucial for their formation (Kwok *et al.,* 2010). Therefore, the hyaluronan synthase (HAS), a transmembrane enzyme synthesizing HA, is expressed by all neurons with PNNs (Weigel *et al.,* 1997; Kwok *et al.,* 2010). The synthesized HA is either cleaved off, or it stays bound to the enzyme, anchoring the PNNs to the neuronal membrane (Kwok *et al.,* 2010). HA enables noncovalent binding of another PNN components: chondroitin sulfate proteoglycans (CSPGs) (Spicer *et al.,* 2003). CSPGs of the PNNs are a part of the lectican family. Lecticans share a common structure

of a globular N-terminal domain and one or two link modules at the C-terminus, which enables them to simultaneously bind other ECM molecules. Thus, lecticans act as ECM organizers (Yamaguchi, 2000). At the central core of the protein, CSPGs carry covalently bound linear chains of glycosaminoglycans (GAGs), comprised of alternating units of N-acetylgalactosamine (GalNAc) and glucuronic acid (GlcA) (Yamaguchi, 2000; Silbert and Sugumaran, 2002). GAGs can be sulfated at several positions, usually at the carbon 2 of the GlcA, and/or carbon 4 and/or carbon 6 of the GalNAc. Various combinations of the sulfated positions add to the structural variability of the CSPGs, which significantly affects their function (Bandtlow and Zimmermann, 2000; Deepa *et al.*, 2002; Maeda *et al.*, 2003). Lecticans are essential compound of PNNs and some of them are exclusively present only in the CNS – neurocan and brevican (Yamada *et al.*, 1994; Watanabe *et al.*, 1995), while others are CNS-unspecific: aggrecan (Acan) and versican (Glumoff *et al.*, 1994; Popp *et al.*, 2003). Acan is always found in the adult PNNs, unlike the remaining CSPGs, as their presence varies across various neuronal subpopulations (Galtrey *et al.*, 2008) and is the main effector of physiological function of PNNs (Rowlands *et al.*, 2018). Production of CSPGs is commenced during embryonal development or shortly after birth and is provided either by neurons, glia, or both (Carulli *et al.*, 2006; Carulli et al., 2007). Besides lecticans, PNNs contain also the CSPGs phosphacan (Maurel *et al.*, 1994), neuroglia 2 (NG2)(Watanabe *et al.*, 1995; Deepa *et al.*, 2006), neuroglycan-C (Oohira *et al.*, 2004), biglycan (Margolis and Margolis, 1997) and decorin (Kappler et al. 1998; Iozzo 1999). As previously mentioned, CSPGs bind also other molecules besides the HA: on the C-terminal end, they bind tenascins, and on the N-terminal end, they bind link proteins. Tenascins are trimeric modular glycoproteins, which allows them to interconnect up to three CSPGs and stabilize the whole structure of PNNs (Lundell *et al.,* 2004). PNN-characteristic tenascin is the tenascin-R (Tn-R)(Tucker and Chiquet-Ehrismann, 2009), which is crucial for normal assembly of the PNNs (Weber *et al.*, 1999). Link proteins are molecules with great binding capacity and are part of the hyaluronan- and proteoglycan-binding link protein gene family (HAPLN) (Spicer *et al.,* 2003). In the PNNs, we find two link proteins: cartilage link protein Crtl1 (HAPLN1) and brain link protein Bral2 (HAPLN4), which are expressed exclusively by neurons with PNNs (Bekku *et al.*, 2003; Rauch *et al.*, 2004; Carulli et al. 2006). Crtl1 is necessary for assembly of diffuse components of PNNs into a condensed net around neurons (Carulli *et al.*, 2010; Kwok *et al.,* 2010).

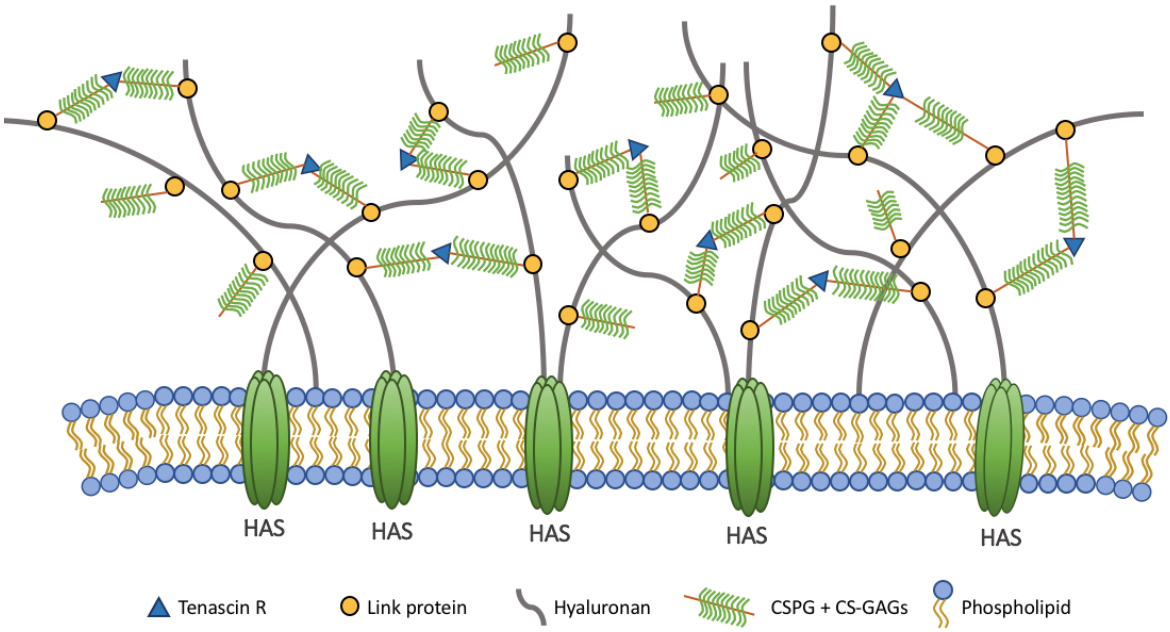

Fig. 1. A scheme of PNN structure. The backbone of the PNNs, HA, is synthesized by HAS. Portion of HA is not cleaved off the HAS, anchoring PNNs to the neuronal membrane.

3.3 The role of PNNs in the critical period

In the course of postnatal development, brain network undergoes maturation, assessed by means of synaptic plasticity: synaptogenesis and remodeling of existing synaptic contacts. Maturation is enabled during a temporal window called the „critical period", characteristic for its increased sensitivity to sensory experience, in order to assess final refinement of neuronal microcircuit patterns. After being exposed to external stimuli during critical period, neurons form appropriately connected microcircuits, which are later stabilized and maintained throughout adulthood (Berardi *et al.,* 2000; Pizzorusso, 2002). Unless there is enough stimuli during critical period, the network lacks important patterns required for normal function (Hensch, 1998, 2005). It is almost impossible to rebuild the ill-formed connectivity later in life. At the close of critical period, neuronal circuitry is stabilized by perineuronal nets developed by the PV+ cells, which results in significant reduction, or even absence, of plasticity.

The importance of critical period and the role of PNNs in restriction of plasticity can be shown on the classical model of visual cortex. Adequate sensory stimuli lead to normal development, while monocular deprivation causes rapid and notable changes in cortical neurons, shifting ocular dominance in favor of the non-deprived eye. Monocular deprivation does not induce the shift in ocular dominance in adult animals. (Guimaraes *et al.,* 1990; Hockfield *et al.*, 1990;

Lander *et al.*, 1997; Pizzorusso, 2002). However, amblyopia in adult rats, caused by monocular deprivation in their youth, can be treated by stimulus-enriched environment, as the increased amount of sensory stimuli decreases density of PNNs and helps to restore visual acuity (Sale *et al.*, 2007).

PNNs form coincidentally with the close of the critical period, as they put brakes on synaptic plasticity and prevent further changes in neuronal network. However, enzymatic degradation of PNNs in the visual cortex of adult organism reopens the critical period window, increasing ocular dominance plasticity to the levels of juvenile brain (Pizzorusso, 2002).

The duration of critical period can be extended by overall reduction of neuronal activity during critical period, as it is linked to late assembly of CSPGs into PNNs and their decreased abundance (Lander *et al.*, 1997). Moreover, sensory deprivation leads to downregulation of Acan expression, which is, conversely, upregulated by depolarization of neurons in vitro (McRae *et al.*, 2007; Giamanco and Matthews, 2012; Ye and Miao, 2013).

The role of PNNs in regulating synaptic plasticity is studied mostly by disruption of their function and formation. To form normal PNNs, the cells need to express at least one of the CSPGs, the one essential being Acan; HAS to form the backbone of the structure and to dock PNNs on the surface of neurons; and a link protein to bind together HA and diffuse CSPGs (Carulli *et al.*, 2010; Kwok *et al.*, 2010; Rowlands *et al.*, 2018). The expression of most of the PNN components starts during embryonal development or several days after birth: Acan, neurocan, HAS and Tn-R are present in the brain by postnatal Day 3. Versican, phosphacan and brevican, produced by glial cells, are present from the time of birth (Carulli *et al.*, 2006; Carulli et al. 2007). However, these components together are not able to condense into PNNs and they are only present in diffuse form, which is not restrictive to synaptic plasticity. Condensation into PNNs is triggered by upregulation of Crtl1, which is necessary to bind together remaining components of PNNs. Knockout of Crtl1 in the brain causes attenuation of PNN formation and Crt1-ko mice remain sensitive to monocular deprivation, as they keep juvenile level of plasticity throughout adulthood (Carulli *et al.*, 2010). Therefore, the condensation of diffuse ECM into PNNs, dependent on expression of Crtl1, is crucial for the closure of critical period and PNNs are the element in control of synaptic plasticity. Normal function of PNNs is impaired also by the knockout of another of PNN components, such as Acan, Tn-R and HAS.

It has been suggested and confirmed that PNNs stabilize neuronal networks and restrict synaptic plasticity. However, the processes underlying their function are not fully understood and neuronal connectivity dynamics are regulated through several mechanisms. Critical period during adulthood is kept closed and synapses stabilized due to interaction of PNNs with various proteins of inhibitory nature, such as semaphorin3A (Sema3A) and Orthodenticle homeobox protein 2 (Otx2)(Wang and Fawcett, 2012; Dick *et al.*, 2013). During development, there are changes in sulfation patterns of CSPGs, attracting the mentioned inhibitory molecules and rendering PNNs more restrictive (Beurdeley *et al.*, 2012; Miyata *et al.*, 2012; Dick *et al.*, 2013; Vo *et al.*, 2013). Moreover, the reticulum of PNNs around synaptic contacts acts as a physical barrier, preventing axonal sprouting and limiting lateral movement of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors on the cell surface (Bradbury, Moon and Popat, 2002; Frischknecht *et al.*, 2009).

3.4 Sulfation patterns of CSPGs

Out of various components of PNNs, their plasticity-restrictive properties are carried out by CSPGs. That is enabled by the presence of binding sites, situated on GAG chains of the CSPGs, and sulfation of their sugars on several positions – specifically the C4S; C6S; C2,6S; and C4,6S. Sulfation of various positions then provides specific patterns and motifs, recognized by molecules binding to these sites (Gama *et al.*, 2006; Miyata *et al.*, 2012). Some of the motifs are prevalent during a specific stage of life, which significantly affects function of PNNs (Kitagawa *et al.*, 1997; Miyata *et al.*, 2012; Foscarin *et al.*, 2017). At birth, the predominant sulfation position is C6S, bearing permissive properties to synaptic plasticity and neurite outgrowth (Lin *et al.*, 2011). Upon the progress of critical period, C6S is gradually depleted and replaced by C4S (Fig. 2), a position acting restrictive to plasticity (Deepa *et al.*, 2006; Carulli *et al.*, 2010; Miyata *et al.*, 2012). Shift in the C4S/C6S ratio is caused by the decreased activity of the chondroitin 6-sulfotransferase-1 (C6ST1) enzyme, sulfating chondroitin on C6, and increased activity of the chondroitin 4-sulfotransferase-1 (C4ST1), promoting sulfation of C4 (Silbert and Sugumaran, 2002; Mikami and Kitagawa, 2013) . At the end of the critical period, 80.6% of CS-GAGs is sulfated at C4 and only 4.9% at C6 (Deepa *et al.*, 2006; Carulli *et al.*, 2010).

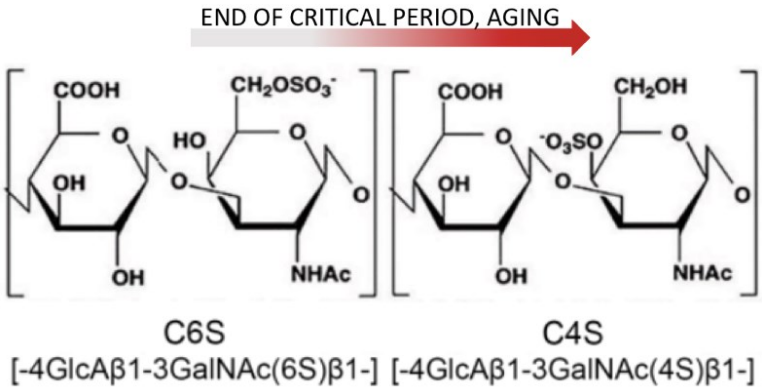

Fig. 2. Transition of predominant sulfation position from C4S co C6S. Upon the end of critical period and during aging, predominant PNN sulfation position is changed from C4S to C6S. (Customized image, original acquired from Foscarin *et al.*, 2017)

The restrictive properties of C4S are dependent on molecules of inhibitory nature, which are attracted to this PNN-characteristic binding site, and their subsequent signaling. Such molecules are Sema3A and Otx2, which both affect the interplay between maturation of inhibitory circuit, critical period and restriction of plasticity in adulthood (Beurdeley *et al.*, 2012; Wang and Fawcett, 2012; Dick *et al.*, 2013).

3.5 Orthodenticle homeobox 2 and Semaphorin 3A

Through the whole life, PV+ interneurons in many areas of the brain require progressive interaction with Otx2, which is an essential factor responsible for two-threshold model of neuronal network maturation (Sugiyama *et al.*, 2008; Miyata *et al.*, 2012). At first, the accumulation of Otx2 is linked to maturation of PV+ neurons and therefore also to the refinement of inhibitory-excitatory balance of neuronal network, which triggers the onset of critical period (Miyata *et al.*, 2012). The second threshold is reached, when PV+ neurons acquire PNNs. Otx2 binds to the C4S chondroitin sulfate position, which enables its subsequent signaling and potentially internalization. (Miyata *et al.*, 2012). Otx2 signaling then acts to make and preserve PNNs in the adult organism and express parvalbumin by the neurons, which creates a positive feedback loop between PNNs attracting Otx2, and their maintenance. This process is partially responsible for closure of critical period and restriction of plasticity in the adulthood. Preventing the neurons from Otx2 internalization prolongs the critical period, as it leads to decrease in PV expression and PNN development in PV+ interneurons (Sugiyama *et al.*, 2008; Sugiyama *et al.,* 2009; Miyata *et al.*, 2012; Spatazza *et al.*, 2013). Otx2 is therefore

crucial for temporal regulation of critical period, proper function of PV+ interneurons and assembly and maintenance of PNNs throughout adulthood.

Sema3A is a signal molecule active during development and it is involved in various processes, such as neuronal migration, growth cone guidance, axon and dendrite growth and branching, axonal transport, and apoptosis (Behar *et al.,* 1996; Taniguchi *et al.*, 1997; Li 2004; Schwarting *et al.*, 2004, 2018). However, in several parts of the CNS, the activity of Sema3A persists into adulthood (Giger *et al.*, 1998), modulating synapse dynamics and neurite outgrowth. In adulthood, Sema3A binds to the PNNs, specifically the 4,6-CS position of CSPGs, probably sharing the similar binding domain with Otx2 (Miyata *et al.*, 2012; Dick *et al.*, 2013; Vo *et al.*, 2013). Thus, PNNs enable the presentation of Sema3A to the cells they ensheath and its interaction with its receptors. Inhibitory effects of Sema3A could be based on local modulation of synaptic dynamics of PV+ cells, as shown in the studies of Sema3A effect on cerebellar and hippocampal circuits (Carulli *et al.*, 2013; Vo *et al.*, 2013).

3.6 PNNs provide cation buffering and oxidative stress protection

Besides representing a leading member in regulating synaptic plasticity, PNNs have also other functions in the adult organism. In the cortex, most of the neurons with PNNs are PV+ interneurons, facilitating fast and rhythmic synchronization and controlling the principal neuron output (Sohal *et al.*, 2009). Therefore, they are known for their fast spiking activity, high metabolic activity and increased sensitivity to oxidative stress, which requires maintaining of local ion homeostasis and protection from reactive oxygen species (ROS) (Sohal *et al.*, 2009; Cabungcal *et al.*, 2013). The PNNs fulfill this task. Sulphate groups of the GAG chains and negatively charged HA give rise to a highly polyanionic microenvironment around the neurons that PNNs ensheath. Thus, PNNs can act as a buffering system interacting with calcium, sodium and potassium, to keep the homeostatic ion balance required for adequate function of PV+ neurons (Brückner *et al.*, 1993; Härtig *et al.*, 1999). Moreover, the polyanionic character of PNNs also allows interaction with ions partially responsible for generating oxidative stress, such as iron. PNNs, most notably its component Acan, are able to bind the redox-active iron and prevent neuronal damage potentially done by harmful oxidative processes (Cabungcal *et al.*, 2013).

3.7 PNNs and memory

Experience-dependent synaptic plasticity can be observed at a notable scale during critical periods of sensory cortex development. However, the changes in synaptic strength are exerted during the entire life – in the processes of learning and memory, which share the same underlying mechanisms of synaptic plasticity (Bliss and Collingridge, 1993; Martin *et al.*, 2000; Martin and Morris, 2002; Griffiths *et al.*, 2008). As the PNNs rather support the state of synaptic stability and reduce the degree of potential strengthening or weakening of synapses, they are involved in control of various forms of memory.

The amygdala is responsible for processing fear conditioning, a memory model that was explored first in relation to PNNs. After the formation of PNNs in the amygdala, conditional fear memories are no longer susceptible to unlearning by extinction, as they were during an early developmental stage in young organism. However, removal of PNNs in the adult, using chABC, enables fear memory relearning or erasure, similar to the juvenile developmental stage. Digestion of PNNs has no effect on conditional fear learning. Therefore, it was suggested that fear memories in the adult are protected by the PNNs (Gogolla *et al.*, 2009). Consistently, removal of hyaluronic acid in the hippocampus results in formation or retrieval impairment of contextual fear memories (Kochlamazashvili *et al.*, 2010), enzymatic degradation of PNNs enables synaptic plasticity enhancement promoting the processes of reversal learning in the tone discrimination task elicited by auditory cortex (Happel *et al.*, 2014), and the genetic ablation of Tn-R in mice leads to improvement in working memory, faster relearning and increased reactivity to novelty (Morellini *et al.*, 2010). These findings inspired the idea to utilize the increase in synaptic plasticity by PNN removal to enhance mechanisms of declarative memory and learning. The declarative memory in rodents is represented by novel object recognition memory, linked to long-term decrease in neuronal responsiveness in perirhinal cortex (Brown *et al.,* 1987; Zhu *et al.*, 1996; Xiang and Brown, 1998), dependent on perirhinal long-term depression (LTD)(Griffiths *et al.*, 2008; Winters *et al.,* 2008) and commonly tested by the spontaneous object recognition task (ORT) (Eichenbaum *et al.,* 2007; Winters *et al.,* 2008).

To investigate the role of PNNs in regulating long-term OR memory, Romberg et al. conducted an experiment combining genetic attenuation and enzymatic degradation of PNNs in murine model (Romberg *et al.*, 2013). The study used the Crtl1-ko model established previously by Carulli and colleagues (Carulli *et al.*, 2010), as the knockout of *hapln-1* gene essential for PNN

function averted formation of PNNs and prolonged the increase in synaptic plasticity into adulthood. Adult Crtl1-ko mice performed significantly better in ORT than the wild type (wt) control group, the difference being especially notable when testing long-term memory. To assess the effect of disrupting CSPG signalisation in normal PNNs, intact in the Crt1-ko model, the control group was injected with the enzyme chABC to acutely remove the PNNs. Treatment by chABC rescued OR memory to the levels of Crtl1-ko mice. The effect could be observed until the PNNs formed again in the perirhinal cortex due to natural turnover and constant expression of their components by neurons and glia (Romberg *et al.*, 2013). Thus, both general genetic attenuation of PNNs and acute enzymatic treatment by chABC improve OR memory in mice. Finally, in a recent study from Rowlands et al. (Rowlands *et al.*, 2018), there was developed a model of conditional Acan knock-out, Acan-ko. Compared to the Crtl-1 ko model, both Acan-ko and Crtl1-ko preclude the formation of PNNs and absence of both Crtl1 and Acan prevent crosslinking of PNN components; however, Acan-ko model is superior in regards to ablate a CSPG essential to PNN function, which enables to prevent not only crosslinking, but also normal formation of CSPG sulfation patterns and their subsequent signalling. Furthermore, immunohistochemistry (IHC) staining showed only partial reduction in PNNs in the Crtl-1 ko, whereas in the Acan-ko the PNNs were absent. Acan-ko model also diminishes the side effects of chABC use, as it targets only PNNs and not overall ECM. This innovative model was established only recently, as it was necessary to overcome the problem of Acan being essential compound of cartilage, and the spatially conditional knockout needed to be developed. Results of the study demonstrate that averting aggregation and loss of function of PNNs have positive impact on synaptic plasticity and OR memory, as the Acan-ko mice performed significantly better in testing the long-term memory using ORT, unlike the control groups (Rowlands *et al.*, 2018).

3.8 Mechanisms underlying memory improvement in the absence of PNNs

It has been demonstrated that PNNs regulate several types of learning and memory, most likely through the GAG chains of their CSPGs. Removal of PNNs affects the degree of PV expression and the activity of PV+ cells. Remarkably, the state of PV+ neurons has been shown to be substantial for synaptic plasticity. The state of „low differentiation“ is characteristic for low expression of PV and enzyme glutamate acid decarboxylase (GAD67), synthetizing inhibitory neurotransmitter gamma-aminobutyric acid (GABA). This promotes structural synaptic plasticity, leading to lowered excitatory/inhibitory synaptic density ratios, decreasing overall inhibitory tone in the cortex and enhancing processes of memory consolidation and retrieval

(Donato *et al.,* 2013). Hippocampal neuronal network is shifted into low-differentiation state particularly during learning, as shown in the task of maze navigation Donato *et al.,* 2013). Conversely, entering the state of high differentiation of PV+ cells, characteristic for high expression of PV and GAD67 and increased excitatory/inhibitory synaptic density ratios, stabilises recently formed memory traces. High-differentiation state is induced upon fear conditioning task, typical for stabilizing the fear memories for entire life (Donato *et al.,* 2013). Low-differentiation state of PV+ population of neurons may be achieved by PNN attenuation, either by enzymatic removal (Balmer, 2016; Lensjø *et al.*, 2017; Rowlands *et al.*, 2018) or genetic ablation, as the Acan-ko mice express less PV (Rowlands *et al.*, 2018).

Perineuronal nets also affect synaptic transmission. Generally, the main mechanisms of synaptic plasticity underlying memory are long-term potentiation (LTP), characteristic for enhancing synaptic transmission by presenting larger numbers of AMPA receptors (AMPAR) on the membrane of postsynaptic receptor. The second essential mechanism does the opposite: LTD is typical for decrement of neuronal responsiveness, linked to internalization of AMPAR from the neuronal membrane. To elicit fast synaptic transmission, it is crucial to exchange desensitized receptors for naïve ones from extrasynaptic sites, which is enabled by receptor lateral mobility (Heine *et al.*, 2008). As shown in the study by R. Frischknecht (Frischknecht *et al.*, 2009), dense ECM ensheating neurons restricts the AMPAR exchange to confined membrane compartments. Treatment of hippocampal slices by Hyase to remove ECM augments the rate of AMPAR exchange, leading to increase in paired-pulse ratio (PPR)(Frischknecht *et al.*, 2009). Moreover, in vitro removal of PNNs by chABC in hippocampal slices affects synaptic transmission both in the form of LTP and LTD (Bukalo *et al.,* 2001). As previously mentioned, object recognition memory, dependent on perirhinal cortex and closely studied in relation to PNNs, relies predominantly on LTD (Griffiths *et al.*, 2008). Crtl1-ko and chABC treated mice exhibit enhanced excitatory synaptic transmission and facilitated induction of LTD in the perirhinal cortex (Romberg *et al.*, 2013).

Furthermore, PNNs play a role in spatial restriction of rebuilding the neuronal network. Enhancement of synaptic plasticity by digestion of PNNs is known to facilitate axonal sprouting and forming new synapses. Application of chABC enables removal of physical barrier to neuronal growth represented by the PNNs and ECM, which contribute to development of glial scar in the injured tissue due to upregulation of CSPGs by glial cells (Smith-Thomas *et al.*, 1995; Asher *et al.*, 2000). This approach is studied to facilitate the treatment of chronic spinal

cord injury (SCI). Remarkably, digestion of PNNs in the spine of rats with SCI, done either by contusion, hemisection or transection, allows axon sprouting and axonal growth across the injured site (Lemons *et al.,* 1999), facilitating better anatomical and functional recovery of these animals (Bradbury *et al.,* 2002; Massey, 2006; Galtrey and Fawcett, 2007; Jefferson et al., 2011).

Together, these findings imply that removal of PNNs brings effects beneficial to memory and learning and neuronal outgrowth, as the absence of PNNs promotes low-differentiation state of PV+ neurons, enables lateral movement and faster exchange of AMPAR and promotes formation of new synapses (Fig. 3), accompanied by facilitated LTD. This mechanism of potential memory enhancement has been tested as a promising tool to rescue memory deficits caused by aging and neurodegeneration.

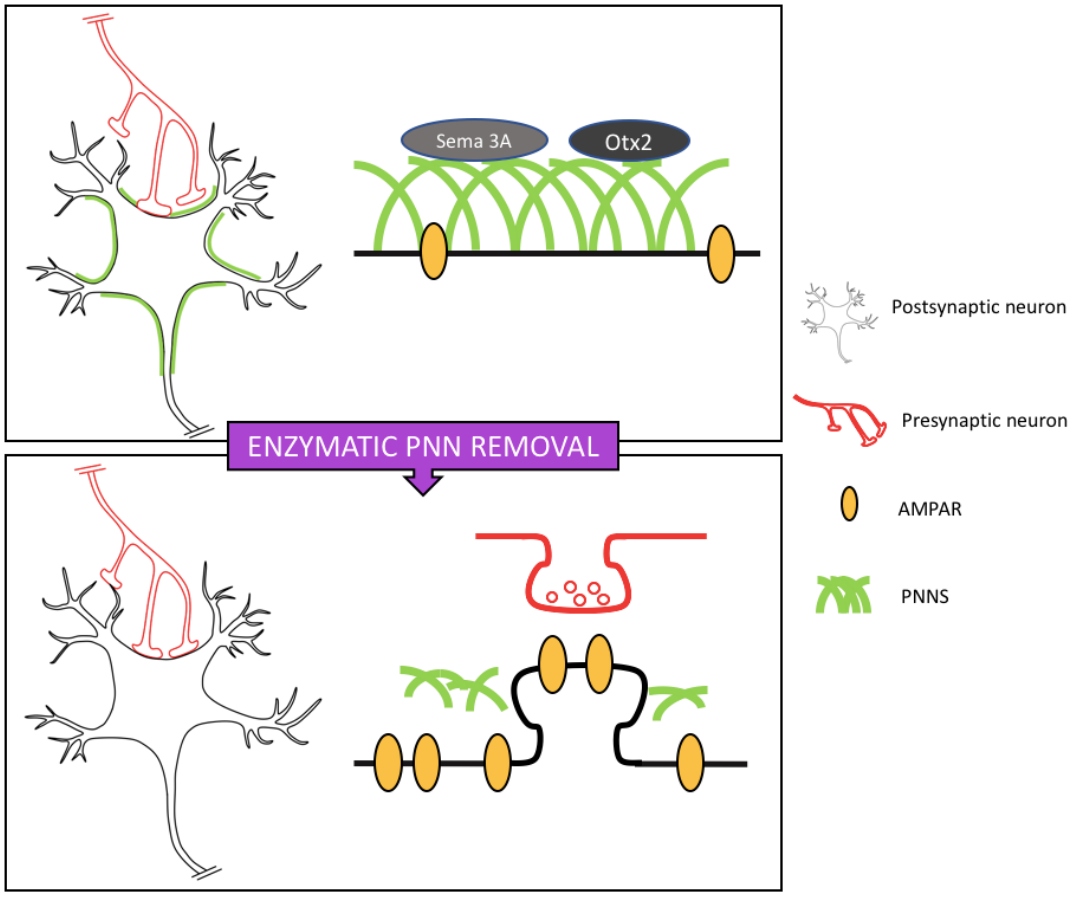

Fig. 3. Beneficial effect of enzymatic PNN removal on synaptic plasticity. In the adult brain, surface of body, proximal dendrites and initial axonal segment is ensheathed by PNNs in PV+ neurons. PNNs bind inhibitory molecules Sema3A and Otx2, reduce AMPAR lateral mobility and restrict synaptic plasticity by posing as a physical barrier to synaptic contact. After enzymatic digestion of PNNs, PNNs no longer bind and present Sema3A and Otx2, allowing the PV+ neurons enter the low-differentiation state, AMPAR lateral mobility is augmented, and there may be formed new neuronal connections.

3.9 PNNs in Alzheimer's disease

During aging, organism is susceptible for gradual decrement of number of synapses, causing progressive loss of memory. This process is also accompanied by change in sulfation pattern of PNNs, as there is an increase in sulfation of C4, a position known to act inhibitory towards synaptic plasticity. Symptoms of aging are amplified upon development of dementia and several neurodegenerative diseases, particularly Alzheimer's disease (AD).

Manifestation of AD is progressive loss of normal cognition and memory and several non-cognitive impairments due to degeneration and loss of neurons. The major risk factor for onset of AD is age; however, cause of the disease is multifactorial and individual, known neuropathological hallmarks being accumulation of beta-amyloid plaques and hyperphosphorylated tau protein. Its origin is also rooted in genetics, it is influenced by lifestyle and environment, and our understanding of the disease is still expanding. However, despite being the most prevalent form of dementia and several decades of research and clinical testing, the developed pharmacologic treatment is still rather symptomatic than suppressing the cause of the disease. Thus, research is taking new directions in exploring possible treatment for AD, and a promising outlook was found in manipulating synaptic plasticity through PNNs.

Indeed, in the beta-amyloid accumulation murine model APP/PS1, early hippocampal memory deficits tested by conditional fear learning were rescued to the levels of wt mice, using acute enzymatic removal of PNNs in the hippocampus by chABC. Enzymatic treatment also reversed physiological deficits of memory, as the chABC-treated APP/PS1 mice demonstrated normal LTP, unlike the group treated with inactive control enzyme penicillinase (Végh *et al.*, 2014). Similar findings were obtained using the mouse strain P301S, characteristic by expression of hyperphosphorylated tau, and murine model, where the pathology is induced by injection of adeno-associated virus (AAV) expressing P301S tau (AAV-P301S) into perirhinal cortex (Yang *et al.*, 2015). Local removal of PNNs in the perirhinal cortex using chABC restored significant synaptic transmission deficit found in the 3 months old P301S mice to above the level of control group. Electrophysiological findings are supported by behavioural testing, as both P301S and AAV-P301S chABC treated animals showed object recognition memory reinstated and comparable to control groups, unlike the untreated group of mice with tau pathology (Yang *et al.*, 2015). Furthermore, the same research team developed PNN-specific antibody Cat316, which binds to the C4S sulfation position of CSPGs, blocking its inhibitory properties on synaptic plasticity and partially attenuating formation of PNNs. Injection of Cat316 into perirhinal cortex of P301S mice normalized short-term object recognition memory,

having the same effect on promoting synaptic plasticity as application of chABC (Yang *et al.*, 2017).

3.10 Abolishing the PNN function

As the research of various features of PNNs proceeded during the past years, there have been developed many ways to remove, attenuate or block the action of PNNs (Fig. 4).
A convenient way to study properties of neuronal network in the absence of PNNs is to establish a knock-out of one or more genes essential for PNN structure: the link proteins Crtl1 (Carulli *et al.*, 2010) and Bral2 (Bekku *et al.*, 2012), CSPGs, most notably conditional brain specific ko of Aggrecan (Rowlands *et al.*, 2018), ko of Tn-R (Weber *et al.*, 1999) and quadruple knockout of Tenascin-C, Tenascin-R, Brevican, and Neurocan (Geissler *et al.*, 2013). Furthermore, there has been established line bearing the knock-out of chondroitin sulphate N-acetylgalactosaminyltransferase-1 (CSGalNAcT-1) enzyme, in order to disrupt CSPG production. However, this knock-out resulted in formation of abnormal, but still structurally distinct, PNNs (Yoshioka *et al.*, 2017). Another way of altering normal formation of PNNs is the overexpression of chondroitin 6-sulfotransferase-1, an enzyme responsible for sulfation of CS-GAGs on C6, resulting in impairment of PNN function (Miyata *et al.*, 2012).

Inhibitory properties of PNNs on synaptic plasticity may be also diminished by blocking the interaction between PNNs and their receptors, receptor protein tyrosine phosphatase σ (RPTPσ)(Shen *et al.*, 2009), leukocyte common antigen-related phosphatase (LAR)(Fisher *et al.*, 2011), and the Nogo receptors (Dickendesher *et al.*, 2012), which, however, have effect mostly on promoting neurite outgrowth. Another method of disrupting the PNN inhibitory signalisation is through blocking the CS-GAG chains by an antibody, specifically the Cat316 (Yang *et al.*, 2017). Critical period can be delayed by introducing a mis-localisation mutation in the *Otx2* (Lee *et al.*, 2017).

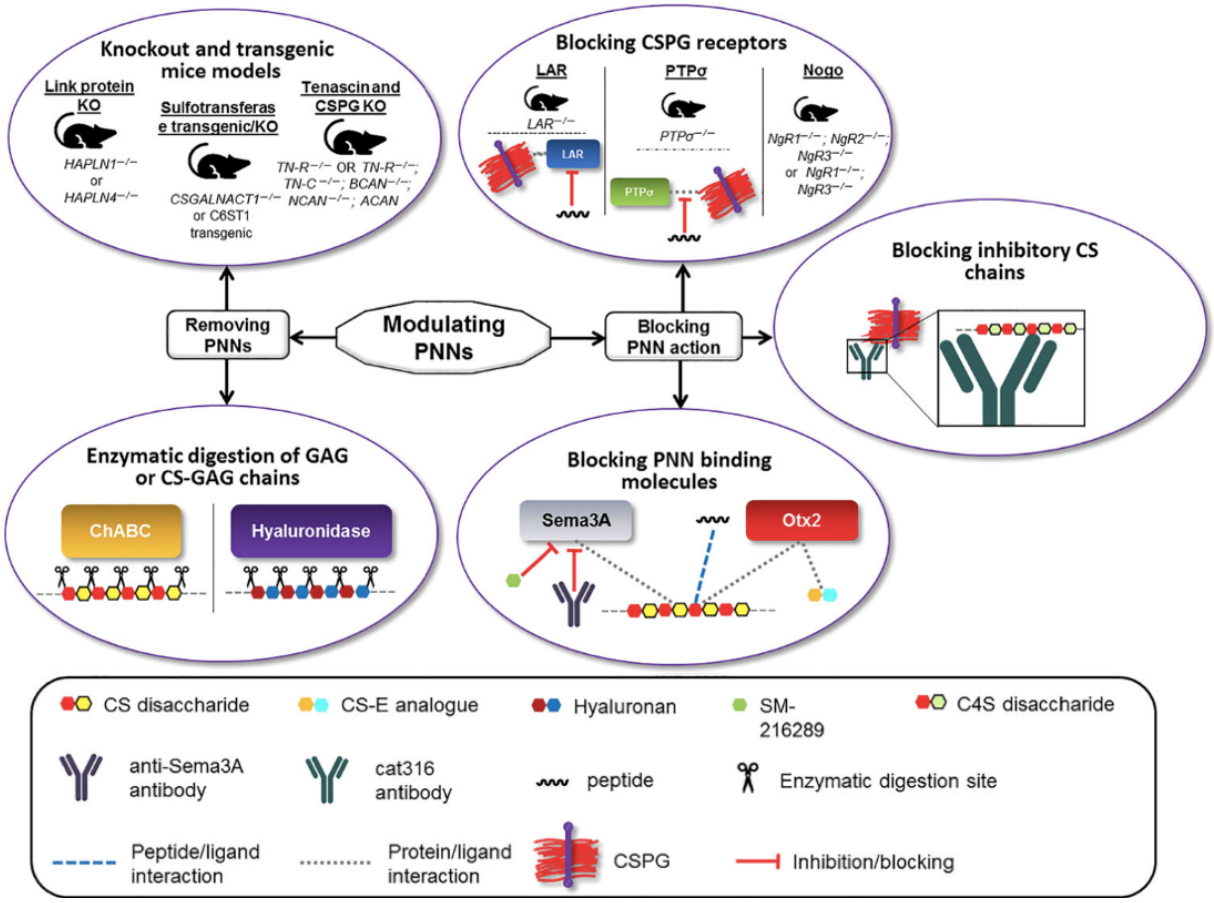

Fig. 4. Graphic summary of methods used to remove and attenuate PNNs or to block their actions. (Image acquired from Duncan, Foster and Kwok, 2019)

To locally remove the PNNs, first method available was application of the enzyme chABC, isolated from *Proteus vulgaris* (Yamagata *et al.,* 1968). Its course of action on PNNs involves endolytic cleavage of beta-1,4-galactosaminic bonds between N-acetyl galactosamine and D-glucuronic acid, indiscriminately digesting CS-GAG chains of CSPGs into disaccharides and oligosaccharides, while not affecting the core protein. The enzyme can also partially digest HA, although it acts on HA at a far lower rate than CS-GAGs. Digestion of CS-GAGs results in structural instability and disintegration of PNNs, which allows for neural outgrowth and increase of synaptic plasticity due to abolition of binding inhibitory molecules to the CS-GAG chains and removing the physical barrier, represented by the whole structure of PNNs. Digestion of PNNs by chABC has been extensively used in PNN research since the 1990's, ranging from studies regarding spinal cord injury, various types of memory, and critical period (Lemons *et al.,* 1999; Bradbury *et al.,* 2002; Pizzorusso, 2002; Gogolla *et al.*, 2009; Romberg *et al.*, 2013).

More recently, another enzyme to locally digest the PNNs has been introduced: Hyase, a β-endoglycosidase which specifically cleaves the β-(1→4) linkage in hyaluronic acid, chondroitin, and chondroitin sulphates (Stern *et al.*, 2007), disrupting the backbone of the PNNs, partially digesting CS and also the surrounding ECM. Hyase has been used for example to facilitate axonal outgrowth in rat nigrostriatal tract (Moon, Asher and Fawcett, 2003), to remove ECM in order to study AMPAR mobility (Frischknecht *et al.*, 2009), to enhance cognitive abilities of Mongolian gerbils (Happel *et al.*, 2014), and in combination with chABC to study involvement of PNNs in fear conditioning (Hylin *et al.*, 2013).

4. Aims of the thesis

Chondroitinase ABC is an enzyme digesting the GAG chains of PNN CSPGs, which has been extensively used to enhance synaptic plasticity and memory in rodent brain. Comparable results have been achieved by the use of Hyase, which digests the PNNs differently than chABC by cleaving the HA backbone and affecting bigger portion of ECM. As there is no study known to directly compare the effects of these enzymes on neuronal network in the brain, the aim of the thesis was to assess the effect of chABC and Hyase on synaptic plasticity in the site of PrC, by conducting bilateral stereotactic injection of these enzymes into PrC. Treated animals were behaviourally tested using the object recognition task to assess the effect of enzymatic degradation of PNNs on learning and memory. Western blot quantitative analysis was performed to measure alteration in protein levels of synaptic apparatus. Lastly, IHC staining has been conducted to visualize PNN removal and to observe gradual return of PNNs onto the surface of PrC neurons. To gain data unbiased by pathology or changes in neuronal network due to age, the study used 3-month-old animals of wt mouse strain. We hypothesized that due to different course of action, Hyase may have more profound effect on PNN digestion than chABC, which may be represented by better performance in the object recognition task, increase in the levels of synaptic proteins, and longer-lasting effect of Hyase on PNN removal, together enhancing synaptic plasticity.

5. Materials and methods

5.1 Experimental animals

In order to test the effect of chABC and Hyase in a neutral background strain with no pathology and no symptoms of aging, we used the mouse strain C57/BL6S (Jackson Laboratory). This strain was previously used to introduce the mutation P301S linked to AD (Yang *et al.,* 2015). The animals were exclusively male and behavioural studies started in 3 months of age. Behavioural testing, described in section 5.2, was conducted in four consecutive cohorts, where each cohort was comprised of two experimental groups and two littermate control groups. Experimental groups were stereotactically injected either by chABC or Hyase, as discussed in section 5.3. In order to test the effect of surgery alone, we performed stereotactical injection of saline in one group of control mice, as saline has no known effect on synaptic plasticity and can be used as an inactive agent. For the remaining control group, only behavioural testing was conducted, and no surgery was performed. The total number of animals in each group goes as follows: chABC n = 11, Hyase n = 12, saline n = 11, no surgical treatment n = 10. Animals were housed as littermates in groups of 2-4, had unrestricted access to food and water and were maintained on a 12 h light/dark cycle. All behavioural tests were conducted during the light phase of the cycle. All of the performed procedures were approved by the ethical committee of the Institute of Experimental medicine, Czech Academy of Sciences.

5.2 Object recognition task

The behavioural testing was conducted in a Y-shaped maze, as described by Bartko et al., (Bartko *et al.*, 2011), and previously used in several studies (Romberg *et al.*, 2013; Yang *et al.*, 2015). The construction of the maze was tailored to maximize mouse's attention to the stimuli; therefore, it has 30 cm high, white opaque walls, and it consists of three arms. First one was used as the start arm, the remaining two arms were used to present objects to the animals. Each arm was 8 cm wide and 16 cm long. In the middle section of the maze, 50 cm above the floor of the apparatus, was mounted a video camera to record the trials. The maze was placed in a specialized room tempered to 22°C and with lights dimmed by translucent curtain to not disturb the mice. The objects used for object recognition task were randomly shaped junk items of dimensions approximately 10 cm × 4 cm × 4 cm. The pairs of the objects were evaluated prior the experiment on an experiment-unrelated group of C57BL/6S mice and chosen to prevent bias in interest of animals to one of the objects. Each test session for a given animal utilised a

different pair of objects (Fig. 5), with the exception of the last session, where it was necessary to repeat the use of the first object, as there was no sixth object pair. Sample and novel objects designated for each session were counterbalanced within and across groups.

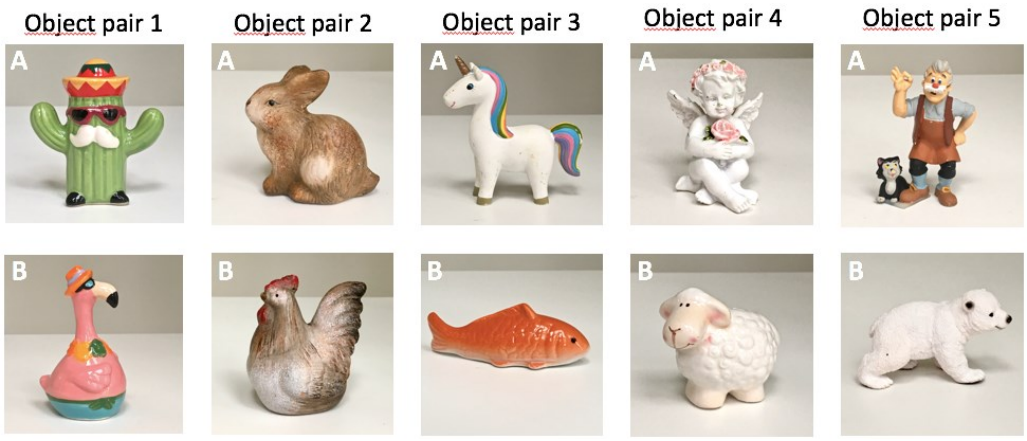

Fig. 5. Five object pairs (A+B) used for the ORT.

The mice were left to explore the empty maze for 5 min in two consecutive sessions, in order to habituate to the apparatus. Habituative and test sessions were separated at least by 48h. Each session of the test consisted of two phases, a sample phase and a choice phase. In the sample phase, the animal is placed in the starting arm, then presented with two identical objects A at the end of each remaining arms, and left to explore the objects for 5 min. Sample phase was followed by delay of 3h or 24h, which the mouse spent in the home cage. After the delay followed the choice phase. The procedure of the choice phase is identical to the protocol of sample phase, except the familiar object A is replaced by a novel object B in one arm (Fig. 6). The other arm contained an unused copy of object A to avoid olfactory bias.

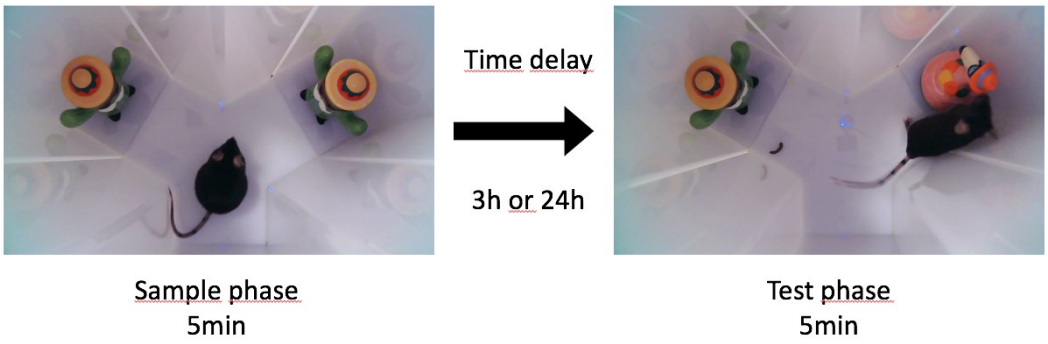

Fig. 6. Experimental setup of ORT. First, the animal participates in the sample phase to explore two identical objects for 5 minutes in a Y-shaped maze. After a time delay of either 3 or 24 hours, test phase is conducted, where the animal is presented with a unused copy of familiar object and a novel object and is allowed to explore the maze for 5 minutes.

Each delay was tested three times for each animal: at the start of the experiment (a week prior to surgery), 1 week after surgery and 3 weeks after surgery (Fig. 7). Object recognition is assessed from measuring exploratory time spent with the familiar and novel object, which is done using the video recordings of sample and choice phase. Only direct head and nasal contact was counted as exploratory behaviour, climbing or sitting on the object was not included. Exploration time of the objects in the choice phase is used to obtain the discrimination ratio, which was calculated by dividing the exploration time of the novel object by the total exploration time. The mean discrimination score across the three test sessions was calculated for each animal. Group means were compared by One-way ANOVA with Tukey's multiple comparisons test, using GraphPad Prism 8.2.0 software.

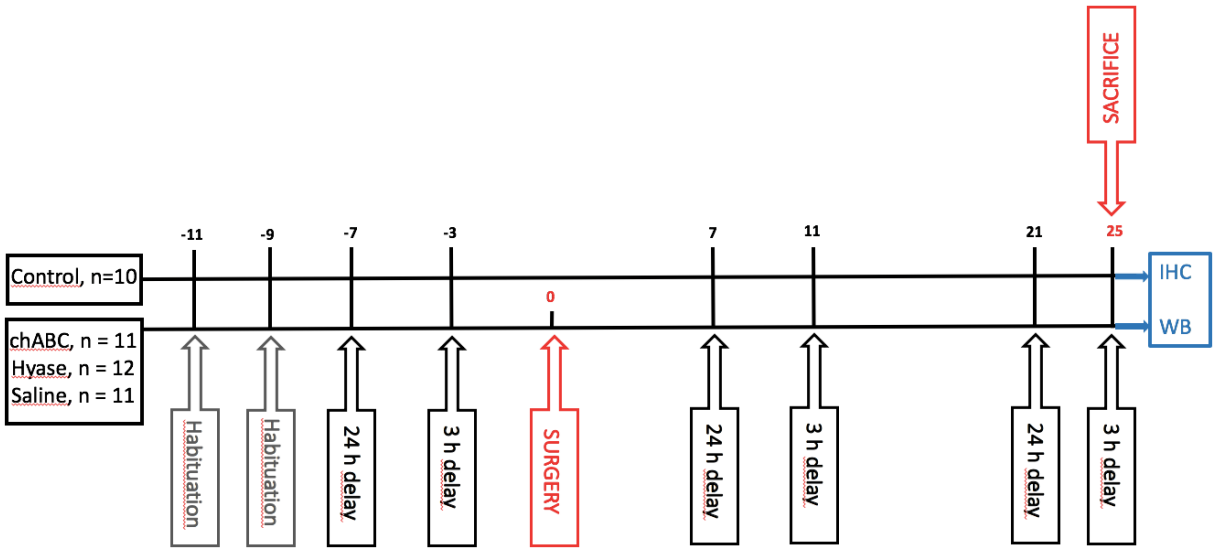

Fig. 7. Timeline of the experiment. All groups of mice went through habituation and behavioural testing of ORT, comprised of three 3h delay paradigm sessions and 24h delay paradigm sessions. The control group of mice did not undergo surgery. At the end of behavioural testing at day 25, all mice were sacrificed, and the brain tissue was used for immunohistochemical staining (IHC) and Western blot (WB) analysis.

5.3 Stereotactic injection

Hyase and chABC were used to digest the PNNs in the perirhinal cortex. Protease-free Hyase or chABC (Seikagaku Kogyo) were dissolved to 50 U/ml in 0.1% BSA. Mice were anesthetized by isoflurane (3% induction, 1,5% maintenance) mixed with oxygen (0,2% flow rate), secured into the stereotaxic frame by ear bars, and put on a heated mat to keep body temperature of animals on constant level, as the body temperature declines during surgery due to anaesthesia. Ophtalmoseptonex was applied to the eyes to prevent damage. Hair above the parietal bones

were cut, surface of the skin was cleaned by ethanol and 1cm long incision was done to expose the skull. To inject the agents, the skull was drilled stereotactically using the motorized Drill and injection robot Stereo Drive (Neurostar GmbH) at six sites in order to access perirhinal cortex. Afterwards, the agents were administered by stereotactic injection into every drilled site (1,5 ul each at 0,2 ul/min rate) using 10 µl Hamilton syringe with a 33 gauge needle (3 per hemisphere, in mm from bregma and the surface of the skull: 1. anterior–posterior (AP): −1.8; lateral (L): ±4.6; ventral (V): −4.4, 2. AP: −2.8; L: ±4.8; V: −4.3 and 3. AP: −3.8; L: ±4.8; V: −3.8), as done in previously conducted studies (Romberg *et al.*, 2013; Yang *et al.*, 2015). Before being slowly withdrawn from the injection site, the needle was left in situ for another 3 minutes to prevent leakage of the agents. The skin incision was closed by polyamide silon monofilament suture and treated by the Novikov solution to facilitate healing of the wound and to prevent infection. The same procedure was conducted for the group of animals, who were administered saline instead of Hyase or chABC. After the surgery, mice were left to recover for 7 days before conducting any further experimental steps.

5.4 Immunohistochemistry

To assess the state of PNNs in the PrC several weeks after injection, we performed immunohistochemical staining. Mice participating in behavioural testing were sacrificed on day 25 of the experiment and therefore can only provide information from three weeks after surgery. To gain information from the whole timeline of the experiment, we unilaterally injected another group of animals with Hyase, chABC and saline, as described in previous section, and these animals were sacrificed 1 week, 2 weeks and 4 weeks post-surgery.

Mice were anesthetized by intraperitoneal injection of ketamine (Narketan 10%, 50mg/kg) mixed with xylazine (Rometar 2%, 6mg/kg) and transcardially perfused with 0.1 M saline, followed by cooled 4% paraformaldehyde (PFA). Brains of the animals were collected and post fixed overnight by PFA, followed by transfer into cryoprotective sucrose gradient (10-30% in 0.2M phosphate buffer). Brains were frozen and cut for IHC staining into coronal slices 20µm thick, using Leica CM1850 cryostat (Leica Microsystems GmbH, Vienna, Austria). Frozen sections were washed 3 times for 5 minutes with PBS (pH = 7,4), followed by washing 10 minutes in 1% H_20_2 and three times for 10 minutes by PBS. After, the slices were washed once for 10 minutes by 0,05% Tween in PBS and blocked for 2 hours in 3% goat serum, followed by staining with primary antibody incubated at 4°C overnight, the *Wisteria floribunda agglutinin* (1:400, L1516, Sigma Aldrich), used commonly to visualize the PNNs.

This was followed by staining with secondary antibody, Streptavidin conjugated with Alexa Fluor 488 (1:400, S32354, Thermo Fisher) for 2 h and DAPI (1:1000, D9564, Sigma Aldrich) for 5-10 min at RT. Images were taken on microscope LEICA CTR 6500 using software FAXS 4.2.6245.1020 (Leica Microsystems).

5.5 Electrophoresis and Western blot analysis

Animals were briefly anesthetized by 2% isoflurane and sacrificed day 25 of the experiment, number of animals being following: chABC n = 6; Hyase n = 4; Saline n = 4; Control n = 7. Brains were quickly dissected and the area of PrC was cut away on a cooled tray. The proteins were isolated using RIPA lysis buffer: (137 mM NaCl, 20 mM Tris (pH 7.8), 1mM MgCl2, 2.7 mM KCl, 1% Triton X-100, 1 mM EDTA, 10% (w/v) glycerol, 1 mM dithiothreitol), which contains phosphatase inhibitor cocktail (Millipore) and protease inhibitor cocktail (Thermo Scientific). Bicinchoninic-acid (BCA) assay (Pierce) was used to determine the total protein concentration of brain tissue homogenate. To conduct western blot analysis, PrC protein samples were separated in 10µg aliquots using 4-15% gradient Mini-PROTEAN TGX Gels (Bio-Rad, cat. no. 456-1083), 50 mA per gel. Protein transfer on PVDF membranes (Life Technologies) was conducted at 350 mA for 60 minutes. Transfer was followed by blocking non-specific background of the membranes with 1 hour incubation in 5% non-fat dry milk (9999S, Cell Signaling Technology) diluted in TBS-T. Afterwards, the membranes were washed three times with TBS-T and incubated with primary antibodies overnight at 4 °C. Primary antibodies included the GAD65/67 antibody, PSD95 antibody, SNAP 25 antibody, VAMP2 antibody, GLUT1 antibody, VGAT antibody and the beta actin antibody (see Tab. 1 for details). Primary antibodies were diluted in the blocking solution. Incubation with primary antibodies was followed by triple washing with TBS-T and incubation for 1h at RT with goat anti-rabbit IgG horseradish peroxidase (1:5000, GE Healthcare UK Ltd.). Afterwards, the membranes were washed three times with TBS-T and were left 1 hour at RT in TBS-T. Each washing step lasted 5 minutes. Visualization of the protein bands was done using the Clarity™ Western ECL Substrate (170-5061, Rio-Rad). Western blot imaging system Azur Biosystems c600 was used to detect the chemiluminescence and visualize the protein bands on the membrane. Quantification of the relative signal intensity of the proteins, using greyscale images, was done with ImageJ software and normalized to the background staining intensity and the positive control protein, β-Actin, staining intensity. Group means were compared by

One-way ANOVA with Tukey's multiple comparisons test, using GraphPad Prism 8.2.0 software.

Tab. 1. List of antibodies used for Western blot analysis.

Antibody	Type	Band size	Concentration	Incubation	Manufacturer	Catalog number
Anti-beta Actin Hrp conjugated	Mouse monoclonal	42	1:8000	Overnight	Abcam	ab49900
Anti-GAD65 + GAD67	Rabbit polyclonal	65/67	1:3000	Overnight	Abcam	ab11070
Anti-PSD95	Rabbit polyclonal	80-85	1:1000	Overnight	Abcam	ab18258
Anti-SNAP25	Rabbit polyclonal	23-26	1:1000	Overnight	Abcam	ab5666
Anti-VAMP2	Rabbit polyclonal	19	1:1000	Overnight	Abcam	ab3347
Anti-VGAT	Rabbit polyclonal	53-57	1:1000	Overnight	Synaptic Systems	131 002
Anti-VGLUT1	Rabbit polyclonal	67	1:1000	Overnight	Synaptic Systems	135 302

6. Results

6.1 Enzymatic digestion of PNNs improves object recognition memory

To assess the effect of enzymatic PNN removal in perirhinal cortex on object recognition memory, we tested 3-month-old wt male C57/BL6S mice injected bilaterally either with chABC or Hyase, and two litter mate control groups, wt mice injected by saline to address the effect of surgery, and wt mice with no surgical treatment. We used the spontaneous object recognition task with delay paradigm of 3 and 24 hours, highly sensitive to OR memory elicited by PrC. Performances of mice in the test were assessed from measurement of exploration times of novel and familiar objects to obtain discrimination ratio. The test was performed one week before the enzyme injection (-1 week), one week after injection (1 week), and three weeks after injection (3 weeks), to assess acute and long-term effect of chABC and Hyase on memory (Fig. 7). All experimental groups performed equally in both 3h and 24h delay paradigms one week before the surgery.

One week after surgery, saline group performed significantly better in the 3h delay paradigm than control group, while chABC and Hyase group showed only mild improvement (Fig. 8). In the 24h delay, control, saline and chABC mice performed equally, with slightly lowered object recognition in Hyase group (Fig. 9). Three weeks after surgery, saline and chABC group showed no recognition memory in the 3h delay paradigm, unlike mice injected by Hyase, who showed a positive difference from the remaining groups (Fig. 8). In the 24h delay paradigm, saline group did not act differently in object recognition task compared to control group, whereas chABC and Hyase mice showed improved performance in the test (Fig. 9).

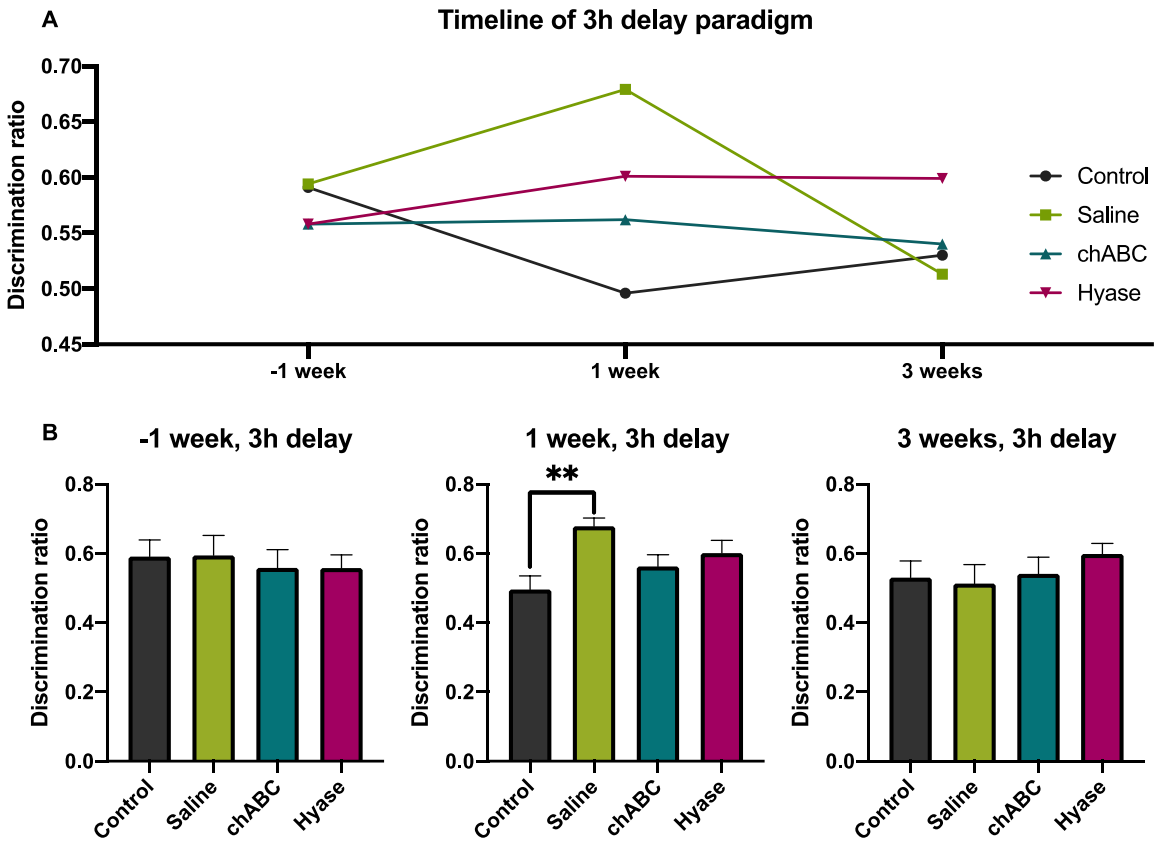

Fig. 8. Results from ORT using the 3h delay paradigm. The figure is showing results in the context of the whole behavioural testing (A) and from the separate sessions (B) conducted one week before surgery, one week post-surgery and three weeks post-surgery (** = $p < 0{,}005$).

Increase or decrease in performance of mice in the object recognition task is unlikely due to different motivation to participate in the test, as all experimental and control groups showed comparable levels of exploration of objects in the sample phase throughout the whole experiment (data not shown). These findings show positive impact of enzymatic removal of PNNs on object recognition memory and suggest that the use of Hyase has longer-lasting effect on object recognition memory than digestion of PNNs by chABC.

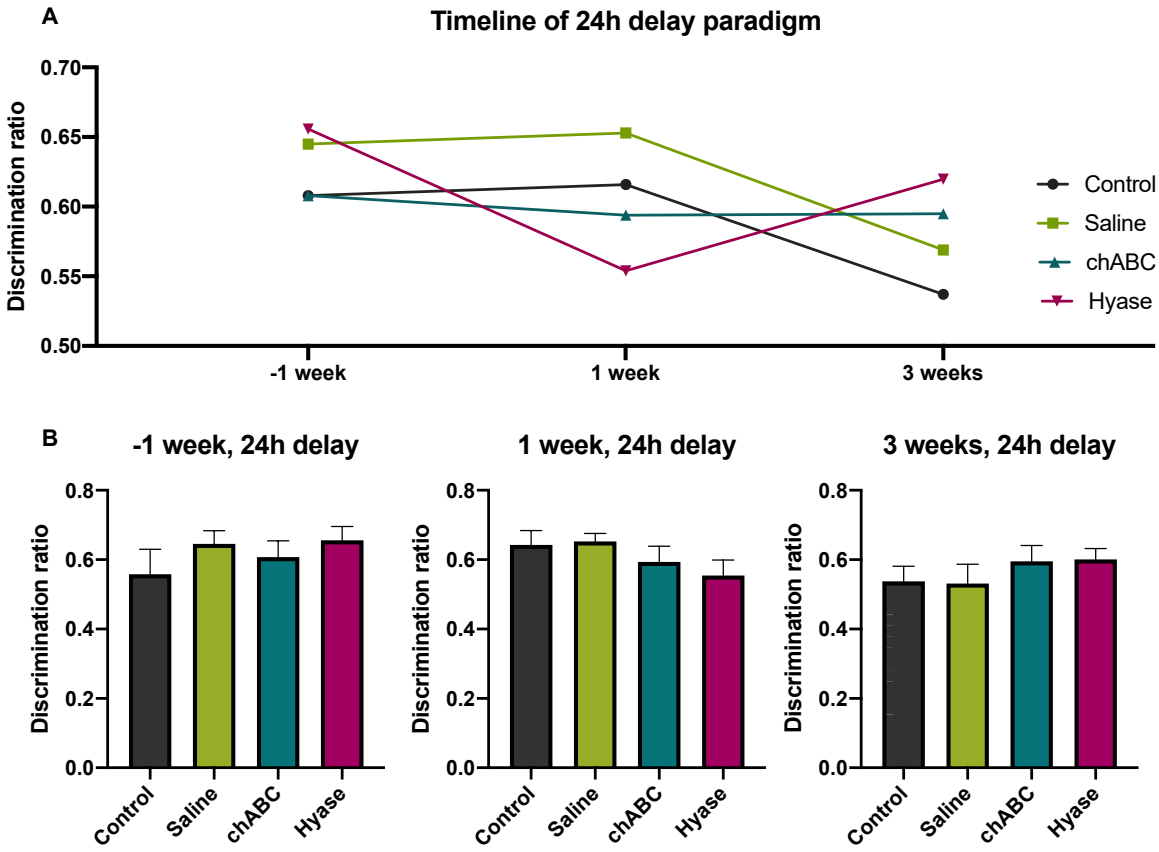

Fig. 9. Results from ORT using the 24h delay paradigm. The figure is showing results in the context of the whole behavioural testing (A) and from the separate sessions (B) conducted one week before surgery, one week post-surgery and three weeks post-surgery.

6.2 Hyase enables to prolong the experimental window of augmented synaptic plasticity

We conducted IHC staining to verify enzymatic removal of PNNs in the site of PrC, and to observe gradual return of PNNs into the brain. To achieve this, we stained coronal brain slices from all groups of animals by WFA, a lectin used to visualize PNNs, and DAPI, and the brains were collected 1 week, 2 weeks, 3 weeks and 4 weeks post-surgery. Afterwards, the area of PrC and surrounding entorhinal cortex was selected from obtained images (Fig. 10) to visually compare intensity of PNNs. PNNs typically surround the soma, dendrites and initial axonal segment of neurons in a well-defined, condensed layer of molecules (Fig. 10). After enzymatic injection, the PNNs are absent, but are gradually regaining their original shape and staining intensity. This takes several weeks, during which the staining is rather diffuse and the PNNs cover mostly the soma and not dendrites of the neuron (Fig. 10).

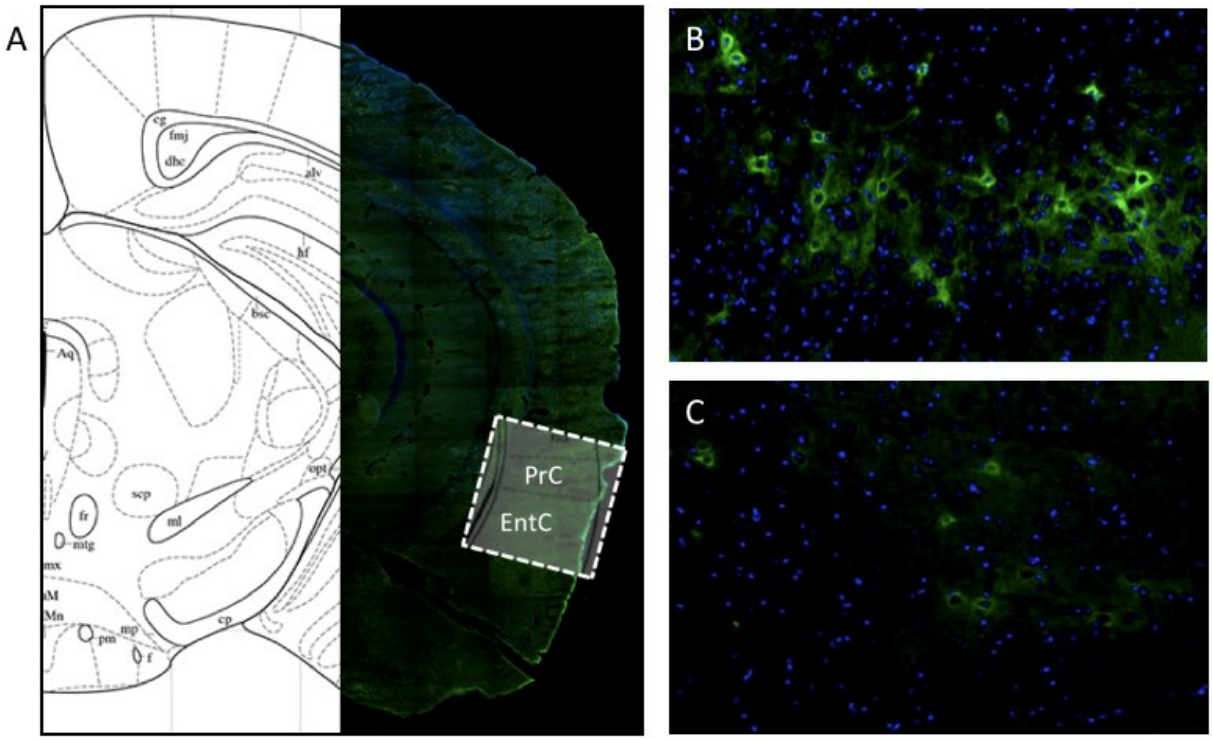

Fig. 10. Examples of the brain area relevant for presentation of the results. Images obtained from IHC staining are cropped in the area of perirhinal cortex (PrC) and surrounding entorhinal cortex (EntC) to provide insight into the site of interest (A). Normal PNNs are characteristic to surround the body, proximal dendrites and initial axonal segment around numerous neurons, and are typical by a well-defined shape and high staining intensity (B). After enzymatic digestion of PNNs, it takes several weeks for the PNNs to reoccur in their original form. Three weeks after injection, neurons in the cortex with removed PNNs are surrounded by diffuse staining of low intensity around small number of neurons (C).

We obtained IHC staining images of PrC from groups of animals injected with Hyase, chABC and saline and from a control group of animals with no surgical treatment. Staining and shape of PNNs in the saline and control group are on the same level throughout the whole experiment, showing that there is no visible effect of saline injection on the PNNs. There are, however, differences in PNN staining in the chABC and Hyase group. One week after surgery, images from these enzymatically treated groups show no presence of PNNs, confirming their digestion by both chABC and Hyase. Two weeks after surgery, the PNNs mostly remain digested, with only weak diffuse WFA staining in both groups and uncondensed PNNs around small number of neurons in the chABC group. Three weeks after surgery, chABC group shows more condensed PNNs present around higher number of neurons, while the Hyase group demonstrates rather diffuse staining around fewer neurons. Major difference is notable in the images obtained four weeks after surgery, as the chABC group shows increase in number of

neurons with PNNs and the PNNs start to ensheath also dendrites, whereas the PNNs of Hyase-injected animal still show intensity and shape comparable to the PNNs seen in images a week prior in the same group. This demonstrates that injection of Hyase causes such a digestion of PNNs, which allows for the longer absence of PNNs compared to injection by chABC.

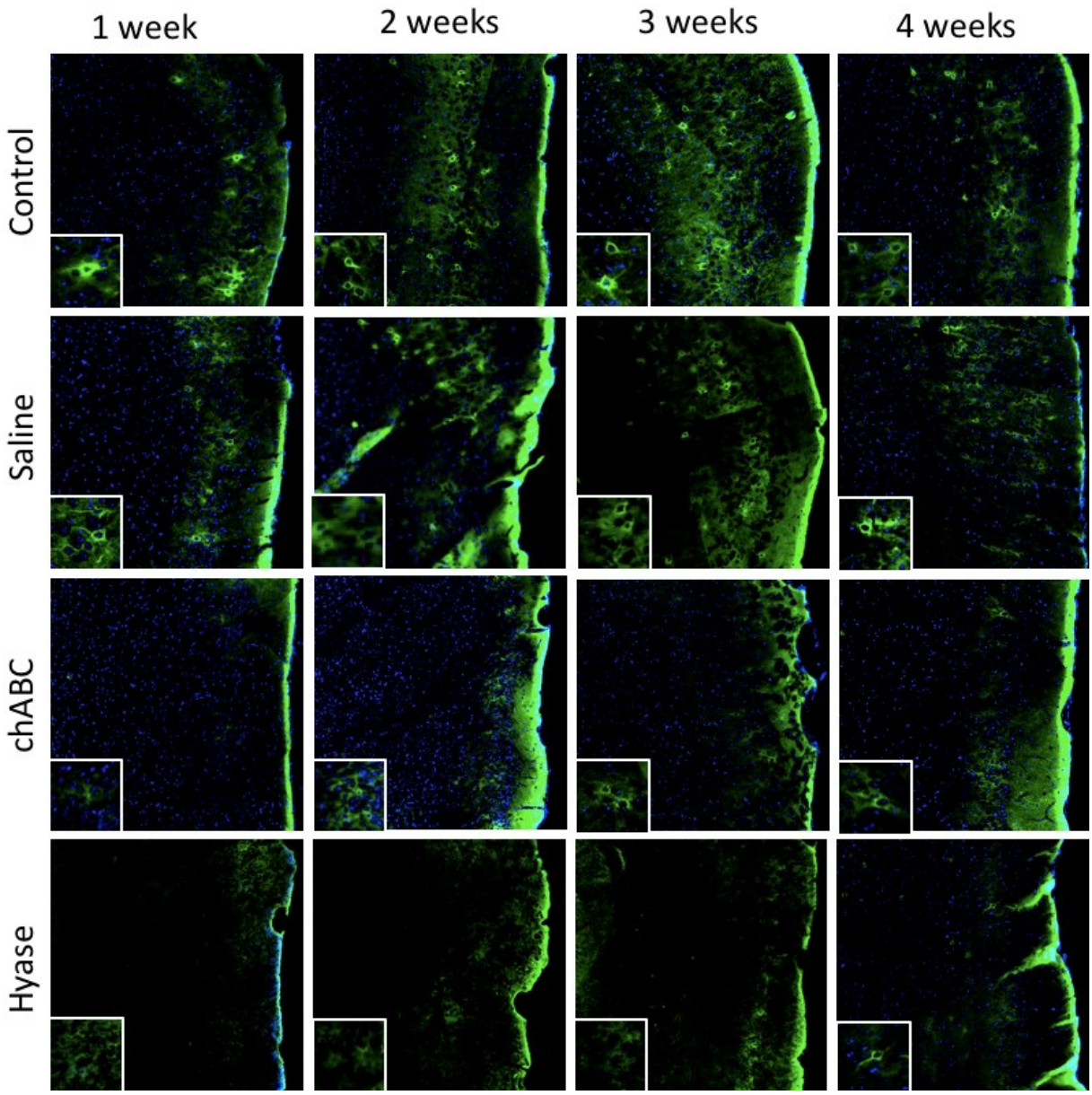

Fig. 11. Immunohistochemical staining of PNNs in the area of PrC. Images were obtained by IHC staining of PrC of the control group and mice injected by saline, chABC, and Hyase, collected one, two, three, or four weeks post-surgery. Images show the area of PrC and EntC and a detail of PNNs from the same image.

6.3 Western blot analysis shows diverse effects of chABC and Hyase on synaptic apparatus

To measure changes in levels of synaptic proteins due to enzymatic removal of PNNs, we conducted quantitative Western blot analysis of samples from PrC of chABC and Hyase treated animals, group of animals with injection of saline and a control group with no treatment. Obtained results were normalized to background density staining and intensity of staining of positive control protein, beta actin, and for the purpose of visualisation extrapolated to saline-injected control group. This group was chosen as the most relevant control in Western blot analysis to eliminate data bias by side-effect changes in protein amounts due to surgery.

6.4 Enhancement of synaptic plasticity prevents damage on synaptic apparatus caused by surgery

To assess overall changes in synaptic activity of neurons affected by enzymatic degradation of PNNs, there was conducted quantitative proteomic analysis of levels of two general synaptic markers. Synaptosomal nerve-associated protein 25 (SNAP25) is a t-SNARE protein essential for the release of neurotransmitter from synaptic vesicles by facilitating fusion of the vesicular and plasmatic membrane of the neuron. Vesicle-associated membrane protein 2 (VAMP2) is another essential component of the same protein complex, participating in the docking and fusion of synaptic vesicles with presynaptic membrane. The data show reduction of SNAP 25 and VAMP2 amounts after injecting mice with saline, demonstrating negative impact of surgery and anaesthesia on neuronal network (Fig. 12). In the case of SNAP25, the decrement in protein level was partially rescued by chABC and Hyase, and the protein level of VAMP2 was rescued and slightly increased in the chABC and Hyase group, indicating that increase in synaptic plasticity after PNN removal is able to surpass and overcome negative effect of injection.

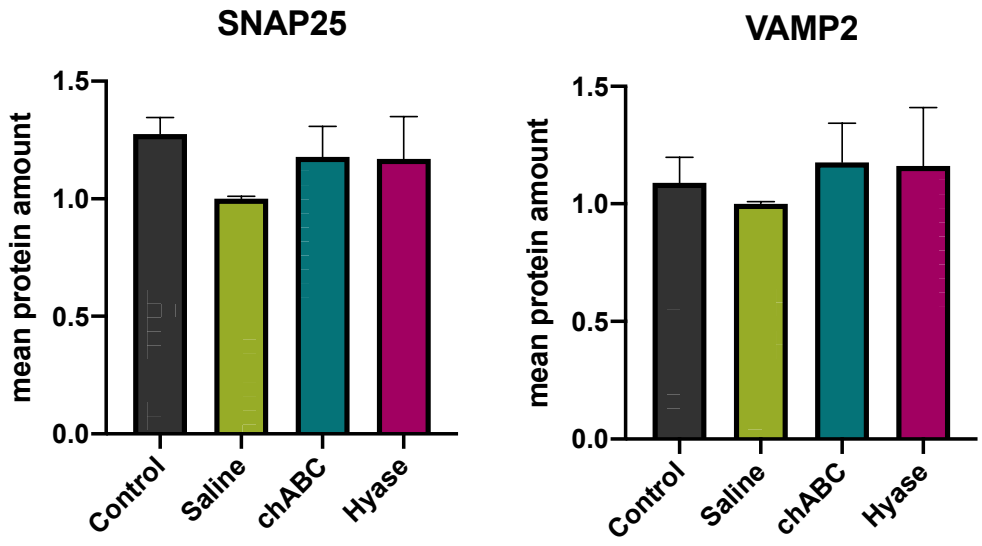

Fig. 12. Levels of general synaptic protein markers, SNAP25 and VAMP2, in the PrC three weeks after digestion of PNNs.

6.5 chABC augments synaptic plasticity of inhibitory neurons

An important marker of activity of inhibitory neurons is the enzyme glutamic acid decarboxylase GAD65/67, which is responsible for catalysing the decarboxylation of glutamate to GABA and CO_2 and in mammals can be found in two isoforms with molecular weights being either 65 or 67 kDa. Vesicular GABA transporter (VGAT) mediates storage of GABA in the synaptic vesicles in the brain. Thus, it was used as another marker of inhibitory neuron synaptic activity. Alterations in activity of inhibitory neurons and release of GABA are known to accompany increase of synaptic plasticity of these neurons. Therefore, we hypothesized an increase in the amount of these markers.

Indeed, the levels of GAD65/67 were heightened in both enzymatically treated groups, more notably in the Hyase group, although the results have no significant value (Fig. 13). The results show significant increase in the levels of VGAT compared to control group in the PrC of mice treated by chABC, as well as nonsignificant increase in the Hyase injected mice (Fig. 13). The obtained data indicate major effect of enzymatic removal of PNNs, especially by chABC, on the increase of amount of proteins involved in the structure and function of inhibitory neurons' synaptic apparatus.

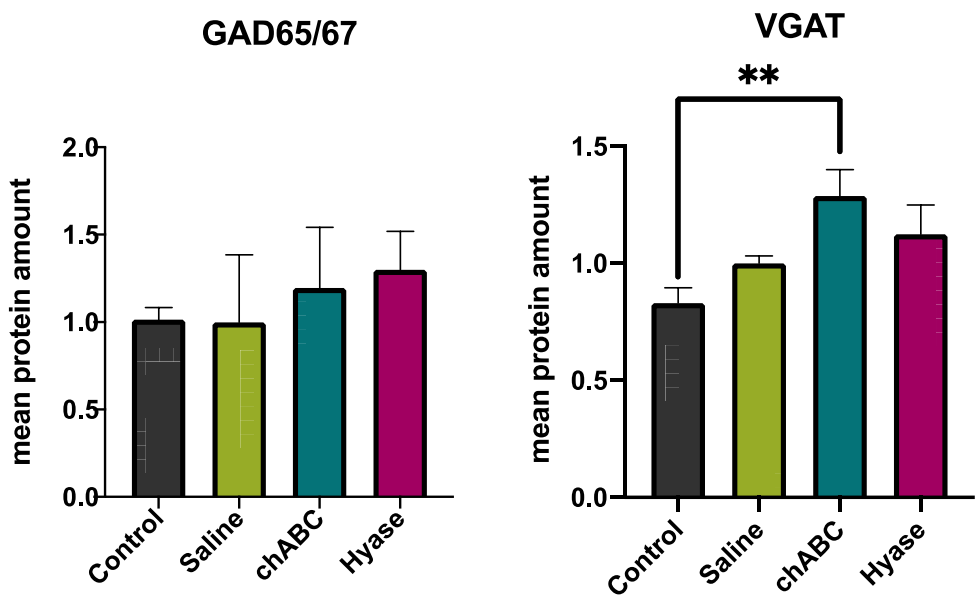

Fig. 13. Levels of synaptic protein markers of inhibitory neurons, GAD56/67 and VGAT, in the PrC three weeks after digestion of PNNs (** = $p < 0{,}005$).

6.6 Hyase further enhances plasticity through effect on excitatory neurons

We conducted analysis of postsynaptic density 95 (PSD95), a postsynaptic scaffold protein, to assess changes on excitatory synapses, as the removal of PNNs alters excitatory/inhibitory balance in the neuronal network. Vesicular glutamate transporter (VGLUT) is a protein facilitating transport of glutamate, neurotransmitter of excitatory neurons, from cell cytoplasm into synaptic vesicles. We analysed amount of this protein in the murine PrC to further explore changes on synapses of excitatory neurons caused by degradation of PNNs by chABC and Hyase.

Quantitative analysis showed significant reduction in PSD95 amount in the saline and chABC group relative to control group with no surgical treatment, indicating invasive effect of surgery and enzymatic degradation of ECM on excitatory neurons (Fig.14).

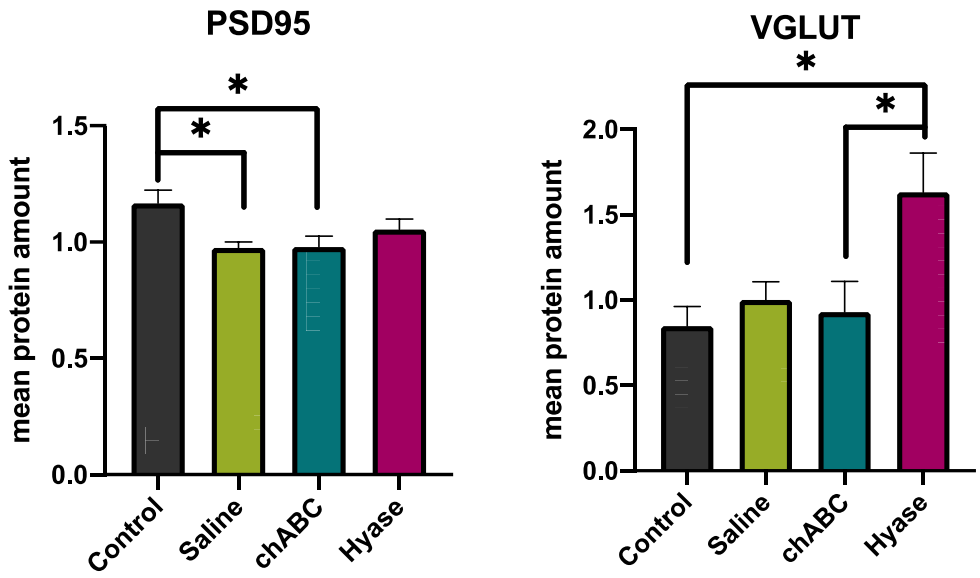

Fig. 14. Levels of synaptic protein markers of excitatory neurons, PSD95 and VGLUT, in the PrC three weeks after digestion of PNNs (* = $p < 0,05$; ** = $p < 0,005$).

On the contrary, reduction of PSD95 in the Hyase group was nonsignificant, indicating that the use of Hyase might be less aggressive than chABC and acute state after surgery is closer to the state of a healthy animal with no treatment. Our findings also show significant increase in levels of VGLUT compared to the control and chABC group, demonstrating that the degradation of Hyase has positive effect on synapses of excitatory neurons in the area of PrC.

7. Discussion

Recently, several studies utilized the enzyme Hyase to digest the PNNs, which takes a distinct course of action enabling to affect bigger portion of ECM than chABC. However, up to this point, there have not been any studies conducted to directly compare features and effects of application of chABC and Hyase on PNN removal and subsequent alteration in properties and function of neuronal network. The aim of this thesis was to digest PNNs by these enzymes at the site of PrC and assess differences in their effect on OR memory and synaptic plasticity. Our results follow up on findings from studies, utilizing chABC to digest PNNs: injection of chABC into PrC improved OR memory in wt mice, accompanied by enhanced LTD underlying OR memory (Romberg *et al.*, 2013). Remarkable improvement of OR memory was observed after chABC treatment of murine model of AD (Yang *et al.*, 2015), as well as improvement of hippocampus-dependent spatial memory in another model of AD (Végh *et al.*, 2014). Hyase has been successfully used to enhance reversal learning in the tone discrimination task in Mongolian gerbils (Happel *et al.*, 2014).

Before discussing data we obtained in our experiments, it is important to point out that the model animals of this study were young, 3-months-old mice with no pathology, and as such possess everything necessary to normal memory function. This state is difficult to surpass, as there is an aim to improve a feature that is already functioning at the best normally possible level. That is reflected in the low differences in the group means both in the data obtained from ORT and WB. However, the experimental design of chABC and Hyase comparison required to be done on a wt model to rule out inevitable bias, which would be caused by pathology or old age. This way, it was possible to observe effects of both enzymes and effect of surgery alone on neutral background. Nevertheless, it would be beneficial to include an experimental group of animals with such impairment to the study, as the method of enzymatic PNN removal has been extensively studied in regards to bring a novel therapeutic method in treating neurodegenerative pathologies and symptoms of aging, and the potential improvement of memory is especially notable in these models.

In our study, we received mixed results from testing the OR memory one week after enzyme injection. In the 3 hours delay paradigm, OR memory of both enzymatic groups surpasses normal levels, indicating successful degradation of PNNs followed by enhancement of learning and memory processes. This improvement in OR memory is more notable in the Hyase group,

although the results did not reach significance threshold. Significant is the improvement of short-term OR memory in the saline group, which highly surpasses the performance of the control group mice, but not the groups of enzyme-injected mice. However, this improvement was only temporary, as the saline group performs on the control level or worse in the 24 hour delay paradigm and in all remaining ORT sessions. We have chosen saline to serve as an inactive agent to observe the effect of injection alone, as we did not expect it to improve or impair OR memory. Thus, the increase observed is unexpected and we consider it an inexplicable exception. For future studies of this kind, we would recommend to use Penicilinase as a control injection agent, as it has some enzymatic activity, possesses features more similar to chABC and Hyase and has been successfully used in previous studies (Romberg *et al.*, 2013; Yang *et al.*, 2015).

In the 24 hours delay paradigm one week after injection, OR memory of ch-ABC treated mice was slightly lowered under the normal performance level, as well as in the Hyase group, where the OR memory is further lowered. These findings are contradicted by results from another study, as the wt mice injected with chABC showed improved OR memory in the 24hour delay one week after injection. We speculate that the performance was affected by a short time between pretraining and surgery, as there was a delay of only three days in our study. Moreover, particularly Hyase cleaved not only PNNs, but also ECM in extracellular space, which could have had an impact on nonsynaptic transmission and changes in tortuosity, leading to worse performance in OR test. However, we cannot compare the exact time between the pretraining and surgery from our study and the study of C. Romberg and team (Romberg *et al.*, 2013), as this study lacks precise specification of the experiment timeline. Our results from the 3h delay (which was performed later after the surgery) is matching the study, as the performance of control and chABC group was on the same level (Romberg *et al.*, 2013).

Three weeks after injection, both enzymatic groups demonstrate improved OR memory compared to control and saline group. The increase in OR memory is observed especially in the Hyase group, being more notable in the 3h delay paradigm compared to chABC. Although our results did not reach significance, they are in line with findings from the study of C. Romberg and colleagues (Romberg *et al.*, 2013), where the application of chABC significantly improved OR memory up to three weeks after surgery and insignificantly up to six weeks after surgery.

Data obtained from ORT are supported by images from immunohistochemically-stained PrC of these animals, which show overall degradation of PNNs one week after surgery both by chABC and Hyase. The absence of WFA staining demonstrates the PNN removal previously

linked to improvement in OR memory, and gradual return of PNNs into the cortex is correlated with return of OR memory on the control level. In our study, we assessed the PNNs in the PrC up to three weeks after surgery, which is the time known to be typical for the PNNs to start to appear in their condensed form and shape but are not yet sufficient to return plasticity on normal level. Thus, there may be observed a decline in OR memory due to gradual emergence of PNNs, but images were still taken in the active stage of enzymatic effect. This decline in OR memory and simultaneous onset of PNN formation is slightly notable in the chABC group, while the Hyase group shows weaker WFA staining around smaller number of neurons even three weeks after the injection. We propose that Hyase has longer-lasting effect on PNN removal, as it digests the backbone of PNN structure, the HA chains anchoring PNNs to neuronal membrane and carrying the CSPGs, but at lower rate also the CS-GAG chains, like chABC does. Therefore, it may digest not only the distinct structure of PNNs condensed tightly around neuronal soma and dendrites and leaving holes for synaptic boutons, but partially also surrounding ECM, as it also contains HA. This way, it is more plausible for the enzyme to affect ECM closer to the synapses and digesting both PNNs and proximal ECM may affect larger portion of neuronal network, since PNNs are carried only by several populations of neurons, namely the PV+ interneurons. Course of action of Hyase may also cause more thorough digestion of PNNs, as chABC only cleaves off the CS-GAG chains of the CSPGs, while Hyase disrupts the PNN structure at its core. Therefore, the ECM around neurons becomes looser, which is one of the features promoting synaptic plasticity, and it may require more time for the neurons to rebuild the PNNs, which is reflected in the later appearance of WFA staining in the PrC of Hyase-treated animals.

Upon the completion of behavioural tests, we conducted quantitative Western blot protein analysis to assess changes in synaptic apparatus of neurons affected by the enzymes. This method has several drawbacks, as it is rather an approximation to the real state of the synapses and to gain more authentic insight, it would be necessary to perform a more elaborate and accurate method, allowing to precisely measure the number and size of newly formed synapses. Such approach has not been included in this study, as it has been used in our parallelly conducted study utilizing the same model and enzymes, where the synapses were analysed by electron microscopy and subdued to counting and measurement. Remarkably, the data obtained from this study are in correlation with the data presented here (J. Růžička unpublished results). It is also necessary to consider that the tissue for WB was collected on the day 25 of the

experiment. Thus, the data reflect the situation from this stage of the study, when the PNNs start to condense around neurons again, and not the acute situation shortly after digestion. Nevertheless, findings from behavioural testing of OR memory are matching with the results from Western blot analysis, which overall demonstrate positive effect of PNN removal by chABC and Hyase on synaptic plasticity, and their different course of action is notable in the alteration of levels of various proteins.

Increase in amount of synaptic protein indicates formation of new synapses or enlarging and strengthening of previously formed synapses. Injection of chABC increased levels of protein markers of synaptic activity of inhibitory neurons, the postsynaptic marker GAD 65/67 and, significantly, the presynaptic marker VGAT. Injection of Hyase also had positive effect on the levels of these markers, especially GAD65/67. Moreover, unlike chABC, application of Hyase significantly increased the level of protein marker characteristic for excitatory neurons, the presynaptic protein VGLUT. Besides VGLUT, we also conducted analysis of another protein marker of excitatory neurons: postsynaptic protein PSD95, where the protein amount was lowered in all groups which went through the surgery, compared to the control group. The decrease is, however, only significant in the saline and chABC group, indicating that injection acts negatively on synapses of excitatory neurons and this negative effect is partially rescued by increased synaptic plasticity instated by Hyase. Decline in PSD-95 levels has been previously described in study from K. Lensjø et al. (Lensjø *et al.*, 2017). Furthermore, we also measured levels of general synaptic protein markers SNAP25 and VAMP2, which demonstrate the same negative effect of surgery, rescued up to some degree by Hyase.

Overall, our data obtained from ORT, IHC staining, and Western blot analysis indicate that there is indeed a beneficial effect on synaptic plasticity resulting from enzymatic degradation of PNNs. Application of chABC proved to improve OR memory and positively affect activity of inhibitory neurons. These findings are not a surprise, as the PV+ interneurons bearing PNNs are known to be involved in regulation of critical period onset and closure and removal of PNNs alters activity of PV+ neurons, accompanied by lowered expression of PV. This may happen due to cleavage of CS-GAG chains, which carried sulfation patterns necessary to act as binding sites for Otx2 and also Sema3A. This way, neurons enter the plasticity-promoting state, facilitating creation of new synaptic puncta. Improvement of OR memory is further promoted by the participation of animals in the ORT itself, as the test acts as a cue to connect and stabilize new synapses. In the situation, where the PNNs are removed, there is an increased potential for

synaptic plasticity; however, new synaptic puncta on pre- and post-synaptic neurons form randomly and they do not have a significant effect to memory improvement. Therefore, it is necessary to present the animal with a positive cue to establish new connections and stabilize them by training. Consequently, increase in spatial synaptic plasticity allows for improved synaptic transmission, which was not analysed in our study, but it is a process underlying the improvement of OR memory according to previously conducted studies.

Moreover, injection of Hyase acts not only on inhibitory interneurons, but according to the data obtained from Western blot analysis, also on excitatory neurons. As a result, it is possible to form more numerous and stable memory traces, which is reflected in better performance of Hyase-injected mice in ORT sessions conducted three weeks after surgery compared to mice injected with chABC.

Although our data lack significance especially in regard to ORT, we believe this is due to a wt murine model we used, and not due to the weakness of this method. On the contrary, we believe it might be useful for future studies to consider the use of Hyase instead of chABC to enzymatically digest PNNs, especially if these studies will be focused on rescuing memory impairment caused by aging or neurodegeneration. Enzymatic removal of PNNs of this design does not pose to be useful in clinical setting due to the need of delivering the enzyme directly into the CNS; however, the scheme of synaptic plasticity enhancement and treatment of memory impairment via degradation of PNNs is promising. Therefore, it would be most useful for future direction of similar studies and especially the clinical use, to develop a method which allows for temporary and controlled digestion of PNNs possible to conveniently instate in a human patient. Gene delivery via adeno associated viruses might be the option.

8. Conclusions

In our study, we have assessed the differences in the effect of enzymatic removal of PNNs either by chABC, or Hyase, on synaptic plasticity and subsequent alterations in OR memory. The mice treated by Hyase showed improved OR memory at three out of four conducted ORT sessions; we found a significant increase in levels of synaptic protein markers of both inhibitory and excitatory neurons, and the images of PrC stained by PNN-binding lectin WFA show slower return of PNNs to their original state. We also succeeded to promote synaptic plasticity by application of chABC followed by improved performance in the ORT and application of this enzyme had profound beneficial effect on synaptic apparatus of PV+ inhibitory interneurons, which is line with previously conducted studies.

Contribution of the thesis to the current state of knowledge lies in direct comparison of the use of chABC and Hyase to digest PNN in the PrC, as there are no studies which would elucidate on this topic, despite the fact that enhancement of synaptic plasticity in this manner has been studied for more than two decades and both of the enzymes have been used to digest ECM in many experiments. As a result, we can recommend the use of Hyase in further studies focusing on the improvement of memory during aging or neurodegenerative diseases. The effect is long-lasting and broadens the therapeutic window. However, further research should also focus on possible side effects of low molecular weight fragments of HA, which may act as signalling molecules and influence the inflammation and synaptic plasticity in pathological states. For possible translation study, the delivery method would have to be changed to gene therapy, most likely utilizing AAV vectors carrying suicide gene.

9. Bibliography

Asher, R. A. *et al.* (2000) 'Neurocan is upregulated in injured brain and in cytokine-treated astrocytes.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 20(7), pp. 2427–2438. doi: https://doi.org/10.1523/JNEUROSCI.20-07-02427.2000

Balmer, T. S. (2016) 'Perineuronal Nets Enhance the Excitability of Fast-Spiking Neurons', *eNeuro*, 3(4). doi: 10.1523/eneuro.0112-16.2016.

Bandtlow, C. E. and Zimmermann, D. R. (2000) 'Proteoglycans in the developing brain: new conceptual insights for old proteins', *Physiol Rev*, 80(4), pp. 1267–1290. doi: 10.1006/dbio.1999.9308.

Bartko, S. J. *et al.* (2011) 'Intact attentional processing but abnormal responding in M 1 muscarinic receptor-deficient mice using an automated touchscreen method', *Neuropharmacology*. Elsevier Ltd, 61(8), pp. 1366–1378. doi: 10.1016/j.neuropharm.2011.08.023.

Behar, O., Golden, J. A., Mashimo, H., Schoen, F. J., & Fishman, M. C. (1996) 'Semaphorin III is needed for normal patterning and growth of nerves, bones and heart.' Nature, 383(6600), pp. 525–528. doi: 10.1038/383525a0

Bekku, Y. *et al.* (2003) 'Molecular cloning of Bral2, a novel brain-specific link protein, and immunohistochemical colocalization with brevican in perineuronal nets', *Molecular and Cellular Neuroscience*, 24(1), pp. 148–159. doi: 10.1016/S1044-7431(03)00133-7.

Bekku, Y. *et al.* (2012) 'Bral2 is indispensable for the proper localization of brevican and the structural integrity of the perineuronal net in the brainstem and cerebellum', *Journal of Comparative Neurology*, 520(8), pp. 1721–1736. doi: 10.1002/cne.23009.

Berardi, N., Pizzorusso, T. and Maffei, L. (2000) 'Critical periods during sensory development', pp. 138–145. doi: 10.1038/nrn848.

Beurdeley, M. *et al.* (2012) 'Otx2 Binding to Perineuronal Nets Persistently Regulates Plasticity in the Mature Visual Cortex', *Journal of Neuroscience*, 32(27), pp. 9429–9437. doi: 10.1523/JNEUROSCI.0394-12.2012.

Bradbury, E. J., Moon, L. D. F. and Popat, R. J. (2002) 'Chondroitinase ABC promotes functional recovery after spinal cord injury', 416(April). doi: 10.1038/416636a.

Brown, M. W., Wilson, F. A. W. and Riches, I. P. (1987) 'Neuronal evidence that inferomedial temporal cortex is more important than hippocampus in certain processes underlying recognition memory', *Brain*, 4119, pp. 158–162. doi: 10.1016/0006-8993(87)90753-0

Brückner, G. *et al.* (1993) 'Perineuronal nets provide a polyanionic, glia-associated form of microenvironment around certain neurons in many parts of the rat brain', *Glia*, 8(3), pp. 183–200. doi: 10.1002/glia.440080306.
Brückner, G., Kacza, J. and Grosche, J. (2004) 'Perineuronal nets characterized by vital labelling, confocal and electron microscopy in organotypic slice cultures of rat parietal cortex and hippocampus', *Journal of Molecular Histology*, 35(2), pp. 115–122. doi: 10.1023/B:HIJO.0000023374.22298.50.
Bukalo, O., Schachner, M. and Dityatev, A. (2001) 'Modification of extracellular matrix by enzymatic removal of chondroitin sulfate and by lack of tenascin-R differentially affects several forms of synaptic plasticity in the hippocampus', *Neuroscience*, 104(2), pp. 359–369. doi: 10.1016/S0306-4522(01)00082-3.
Cabungcal, J.-H. *et al.* (2013) 'Perineuronal nets protect fast-spiking interneurons against oxidative stress.', *Proceedings of the National Academy of Sciences of the United States of America*, 110(22), pp. 9130–5. doi: 10.1073/pnas.1300454110.
Carulli, D. *et al.* (2006) 'Composition of perineuronal nets in the adult rat cerebellum and the cellular origin of their components', *Journal of Comparative Neurology*, 494(4), pp. 559–577. doi: 10.1002/cne.20822.
Carulli D, Rhodes KE, Fawcett JW. (2007) 'Upregulation of aggrecan, link protein 1, and hyaluronan synthases during formation of perineuronal nets in the rat cerebellum.', *J Comp Neurol*. 2007; 501, pp 83–94. doi: 10.1002/cne.21231
Carulli, D. *et al.* (2010) 'Animals lacking link protein have attenuated perineuronal nets and persistent plasticity', *Brain*, 133(8), pp. 2331–2347. doi: 10.1093/brain/awq145.
Carulli, D. *et al.* (2013) 'Modulation of semaphorin3A in perineuronal nets during structural plasticity in the adult cerebellum', *Molecular and Cellular Neuroscience*. Elsevier B.V., 57, pp. 10–22. doi: 10.1016/j.mcn.2013.08.003.
Celio, M. R. and Blumcke, I. (1994) 'Perineuronal nets - a specialized form of extracellular matrix in the adult nervous system', *Brain Research Reviews*, 19(1), pp. 128–145. doi: 10.1016/0165-0173(94)90006-X.
Deepa, S. S. *et al.* (2002) 'Specific molecular interactions of oversulfated chondroitin sulfate E with various heparin-binding growth factors: Implications as a physiological binding partner in the brain and other tissues', *Journal of Biological Chemistry*, 277(46), pp. 43707–43716. doi: 10.1074/jbc.M207105200.
Deepa, S. S. *et al.* (2006) 'Composition of perineuronal net extracellular matrix in rat brain: A different disaccharide composition for the net-associated proteoglycans', *Journal of*

Biological Chemistry, 281(26), pp. 17789–17800. doi: 10.1074/jbc.M600544200.
Dick, G. *et al.* (2013) 'Semaphorin 3A binds to the perineuronal nets via chondroitin sulfate type E motifs in rodent brains', *Journal of Biological Chemistry*, 288(38), pp. 27384–27395. doi: 10.1074/jbc.M111.310029.
Dickendesher, T. L. *et al.* (2012) 'NgR1 and NgR3 are receptors for chondroitin sulfate proteoglycans', *Nature Neuroscience*, 15(5), pp. 703–712. doi: 10.1038/nn.3070.
Donato, F., Rompani, S. B. and Caroni, P. (2013) 'Parvalbumin-expressing basket-cell network plasticity induced by experience regulates adult learning', *Nature*. Nature Publishing Group, 504(7479), pp. 272–276. doi: 10.1038/nature12866.
Duncan, J. A., Foster, R. and Kwok, J. C. F. (2019) 'The potential of memory enhancement through modulation of perineuronal nets', *British Journal of Pharmacology*.
2019, pp. 1–11. doi: 10.1111/bph.14672.
Eichenbaum, H., Yonelinas, A. P. and Ranganath, C. (2007) 'The Medial Temporal Lobe and Recognition Memory', *Annual Review of Neuroscience*, 30(1), pp. 123–152.
doi: 10.1146/annurev.neuro.30.051606.094328.
Fawcett, J. W. (2015) *The extracellular matrix in plasticity and regeneration after CNS injury and neurodegenerative disease*. 1st edn, *Progress in Brain Research*. 218, pp. 213-226.
doi: 10.1016/bs.pbr.2015.02.001.
Fisher, D. *et al.* (2011) 'Leukocyte Common Antigen-Related Phosphatase Is a Functional Receptor for Chondroitin Sulfate Proteoglycan Axon Growth Inhibitors', *Journal of Neuroscience*, 31(40), pp. 14051–14066. doi: 10.1523/jneurosci.1737-11.2011.
Foscarin, S. *et al.* (2017) 'Brain ageing changes proteoglycan sulfation, rendering perineuronal nets more inhibitory', *Aging*, 9(6), pp. 1607–1622. doi: 10.18632/aging.101256.
Frischknecht, R. *et al.* (2009) 'Brain extracellular matrix affects AMPA receptor lateral mobility and short-term synaptic plasticity', *Neuroforum*. Nature Publishing Group, 15(3), pp. 94–95. doi: 10.1038/nn.2338.
Galtrey, C. M. *et al.* (2008) 'Distribution and synthesis of extracellular matrix proteoglycans, hyaluronan, link proteins and tenascin-R in the rat spinal cord', *European Journal of Neuroscience*, 27(6), pp. 1373–1390. doi: 10.1111/j.1460-9568.2008.06108.x.
Galtrey, C. M. and Fawcett, J. W. (2007) 'The role of chondroitin sulfate proteoglycans in regeneration and plasticity in the central nervous system', *Brain Research Reviews*, 54(1), pp. 1–18. doi: 10.1016/j.brainresrev.2006.09.006.
Gama, C. I. *et al.* (2006) 'Sulfation patterns of glycosaminoglycans encode molecular recognition and activity', *Nature Chemical Biology*, 2(9), pp. 467–473.

doi: 10.1038/nchembio810.
Geissler, M. *et al.* (2013) 'Primary Hippocampal Neurons, Which Lack Four Crucial Extracellular Matrix Molecules, Display Abnormalities of Synaptic Structure and Function and Severe Deficits in Perineuronal Net Formation', *Journal of Neuroscience*, 33(18), pp. 7742–7755. doi: 10.1523/JNEUROSCI.3275-12.2013.
Giamanco, K. A. and Matthews, R. T. (2012) 'Deconstructing the perineuronal net: Cellular contributions and molecular composition of the neuronal extracellular matrix', *Neuroscience*. 218, pp. 367–384. doi: 10.1016/j.neuroscience.2012.05.055.
Giger, R. J. *et al.* (1998) 'Anatomical distribution of the chemorepellent semaphorin III/collapsin- 1 in the adult rat and human brain: Predominant expression in structures of the olfactory-hippocampal pathway and the motor system', *Journal of Neuroscience Research*, 52(1), pp. 27–42.
doi: 10.1002/(SICI)1097-4547(19980401)52:1<27::AID-JNR4>3.0.CO;2-M.
Glumoff, V. *et al.* (1994) 'Analysis of aggrecan and tenascin gene expression in mouse skeletal tissues by Northern and in situ hybridization using species specific cDNA probes', *BBA - Gene Structure and Expression*, 1219(3), pp. 613–622.
doi: 10.1016/0167-4781(94)90220-8.
Gogolla, N. *et al.* (2009) 'Perineuronal Nets Protect Fear Memories from Erasure', *Science*, 325(5945), pp. 1258–1261. doi: 10.1126/science.1174146.
Griffiths, S. *et al.* (2008) 'Expression of Long-Term Depression Underlies Visual Recognition Memory', *Neuron*, 58(2), pp. 186–194. doi: 10.1016/j.neuron.2008.02.022.
Guimaraes, A., Zaremba, S. and Hockfield, S. (1990) 'Molecular and morphological changes in the cat lateral geniculate nucleus and visual cortex induced by visual deprivation are revealed by monoclonal antibodies Cat-304 and Cat-301', *The Journal of Neuroscience*, 10(9), pp. 3014–3024. doi: 10.1523/jneurosci.10-09-03014.1990.
Happel, M. F. K. *et al.* (2014) 'Enhanced cognitive flexibility in reversal learning induced by removal of the extracellular matrix in auditory cortex', 111(7), pp. 2800–2805.
doi: 10.1073/pnas.1310272111.
Härtig, W. *et al.* (1999) 'Cortical neurons immunoreactive for the potassium channel Kv3.1b subunit are predominantly surrounded by perineuronal nets presumed as a buffering system for cations', *Brain Research*, 842, pp. 15–29. doi: 10.1057/9781137315304.0026.
Hausen, D. *et al.* (1996) 'Pyramidal cells ensheathed by perineuronal nets in human motor and somatosensory cortex', *NeuroReport*, 7(11), pp. 1725–1729.
doi: 10.1097/00001756-199607290-00006.

Hensch, T. K. (1998) ‘Local GABA Circuit Control of Experience-Dependent Plasticity in Developing Visual Cortex’, *Science*, 282(5393), pp. 1504–1508. doi: 10.1038/098448b0.
Hensch, T. K. (2005) ‘Critical period plasticity in local cortical circuits.’, *Nature reviews. Neuroscience*, 6(11), pp. 877–88. doi: 10.1038/nrn1787.
Hockfield, S. *et al.* (1990) ‘Expression of Neural Proteoglycans Correlates with the Acquisition of Mature Neuronal Properties in the Mammalian Brain’, *Cold Spring Harbor Symposia on Quantitative Biology*, 55(0), pp. 505–514. doi: 10.1101/sqb.1990.055.01.049.
Hockfield, S. and McKay, R. D. (1983) ‘A surface antigen expressed by a subset of neurons in the vertebrate central nervous system.’, *Proceedings of the National Academy of Sciences of the United States of America*, 80(September), pp. 5758–5761.
doi: 10.1073/pnas.80.18.5758.
Hylin, M. J. *et al.* (2013) ‘Disruption of the perineuronal net in the hippocampus or medial prefrontal cortex impairs fear conditioning’, *Learning and Memory*, 20(5), pp. 267–273.
doi: 10.1101/lm.030197.112.
Iozzo, R. V (1999) ‘The Biology of the Small Leucine-rich Proteoglycans’, 274(27), pp. 18843–18846. doi: 10.1074/jbc.274.27.18843.
Jefferson, S. C., Tester, N. J. and Howland, D. R. (2011) ‘Chondroitinase ABC Promotes Recovery of Adaptive Limb Movements and Enhances Axonal Growth Caudal to a Spinal Hemisection’, *Journal of Neuroscience*, 31(15), pp. 5710–5720.
doi: 10.1523/JNEUROSCI.4459-10.2011.
Meyer, K., Linker, A. and Maurice, R. M. (1951) ‘The production of monosaccharides from hyaluronic acid by b-glucuronidase’, *J. Biol. Chem.*, 192, pp. 275–281.
Kappler, J. *et al.* (1998) ‘Developmental regulation of decorin expression in postnatal rat brain’, *Brain Research*, 793(1–2), pp. 328–332. doi: 10.1016/S0006-8993(98)00260-1.
Kitagawa, H. *et al.* (1997) ‘Developmental regulation of the sulfation profile of chondroitin sulfate chains in the chicken embryo brain’, *Journal of Biological Chemistry*, 272(50), pp. 31377–31381. doi: 10.1074/jbc.272.50.31377.
Kochlamazashvili, G. *et al.* (2010) ‘The extracellular matrix molecule hyaluronic acid regulates hippocampal synaptic plasticity by modulating postsynaptic L-type Ca2+ channels’, *Neuron*. 67(1), pp. 116–128. doi: 10.1016/j.neuron.2010.05.030.
Kwok, J. C. F. *et al.* (2011) ‘Extracellular matrix and perineuronal nets in CNS repair’, *Developmental Neurobiology*, 71(11), pp. 1073–1089. doi: 10.1002/dneu.20974.
Kwok, J. C. F., Carulli, D. and Fawcett, J. W. (2010) ‘In vitro modeling of perineuronal nets: Hyaluronan synthase and link protein are necessary for their formation and integrity’, *Journal*

of Neurochemistry, 114(5), pp. 1447–1459. doi: 10.1111/j.1471-4159.2010.06878.x.
Lander, C. *et al.* (1997) 'A family of activity-dependent neuronal cell-surface chondroitin sulfate proteoglycans in cat visual cortex.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 17(6), pp. 1928–39.
https://doi.org/10.1523/JNEUROSCI.17-06-01928.1997
Lau, L. W. *et al.* (2013) 'Pathophysiology of the brain extracellular matrix: A new target for remyelination', *Nature Reviews Neuroscience*. 14(10), pp. 722–729. doi: 10.1038/nrn3550.
Lee, H. H. C. *et al.* (2017) 'Genetic Otx2 mis-localization delays critical period plasticity across brain regions', *Molecular Psychiatry*, 22(5), pp. 680–688. doi: 10.1038/mp.2017.1.
Lemons, M. L., Howland, D. R. and Anderson, D. K. (1999) 'Chondroitin sulfate proteoglycan immunoreactivity increases following spinal cord injury and transplantation.', *Experimental neurology*, 160(1), pp. 51–65. doi: 10.1006/exnr.1999.7184.
Lensjø, K. K. *et al.* (2017) 'Differential Expression and Cell-Type Specificity of Perineuronal Nets in Hippocampus, Medial Entorhinal Cortex, and Visual Cortex Examined in the Rat and Mouse', *Eneuro*, 37(5), pp. 1269-1283. doi: 10.1523/eneuro.0379-16.2017.
Li, C. (2004) 'Correlation between Semaphorin3A-Induced Facilitation of Axonal Transport and Local Activation of a Translation Initiation Factor Eukaryotic Translation Initiation Factor 4E', *Journal of Neuroscience*, 24(27), pp. 6161–6170.
doi: 10.1523/jneurosci.1476-04.2004.
Lin, R. *et al.* (2011) '6-Sulphated chondroitins have a positive influence on axonal regeneration', *PLoS ONE*, 6(7). doi: 10.1371/journal.pone.0021499.
Lundell, A. *et al.* (2004) 'Structural basis for interactions between tenascins and lectican C-type lectin domains: Evidence for a crosslinking role for tenascins', *Structure*, 12(8), pp. 1495–1506. doi: 10.1016/j.str.2004.05.021.
M. Heine *et al.* (2008) 'Surface Mobility of Postsynaptic AMPARs Tunes Synaptic Transmission', *Science*, 320(5873), pp. 201–205. doi: 10.1126/science.1152089.
Maeda, N. *et al.* (2003) 'Heterogeneity of the chondroitin sulfate portion of phosphacan/6B4 proteoglycan regulates its binding affinity for pleiotrophin/heparin binding growth-associated molecule', *Journal of Biological Chemistry*, 278(37), pp. 35805–35811.
doi: 10.1074/jbc.M305530200.
Margolis, R. U. and Margolis, R. K. (1997) 'Chondroitin sulfate proteoglycans as mediators of axon growth and pathfinding', *Cell and Tissue Research*, 290(2), pp. 343–348.
doi: 10.1007/s004410050939.
Martin, S. J., Grimwood, P. D. and Morris, R. G. M. (2000) 'Synaptic Plasticity and Memory:

An Evaluation of the Hypothesis', *Ann.Rev.Neurosci*, 23, pp. 649–711. doi: 10.1146/annurev.neuro.23.1.649

Martin, S. J. and Morris, R. G. M. (2002) 'New life in an old idea: The synaptic plasticity and memory hypothesis revisited', *Hippocampus*, 12(5), pp. 609–636. doi: 10.1002/hipo.10107.

Massey, J. M. (2006) 'Chondroitinase ABC Digestion of the Perineuronal Net Promotes Functional Collateral Sprouting in the Cuneate Nucleus after Cervical Spinal Cord Injury', *Journal of Neuroscience*, 26(16), pp. 4406–4414. doi: 10.1523/JNEUROSCI.5467-05.2006.

Maurel, P. *et al.* (1994) 'Phosphacan, a chondroitin sulfate proteoglycan of brain that interacts with neurons and neural cell-adhesion molecules, is an extracellular variant of a receptor-type protein tyrosine phosphatase.', *Proceedings of the National Academy of Sciences of the United States of America*, 91(7), pp. 2512–2516. doi: 10.1073/pnas.91.7.2512.

McRae, P. A. *et al.* (2007) 'Sensory Deprivation Alters Aggrecan and Perineuronal Net Expression in the Mouse Barrel Cortex', *Journal of Neuroscience*, 27(20), pp. 5405–5413. doi: 10.1523/jneurosci.5425-06.2007.

McRae, P. A. and Porter, B. E. (2012) 'The perineuronal net component of the extracellular matrix in plasticity and epilepsy', *Neurochemistry International*. 61(7), pp. 963–972. doi: 10.1016/j.neuint.2012.08.007.

Mikami, T. and Kitagawa, H. (2013) 'Biosynthesis and function of chondroitin sulfate', *Biochimica et Biophysica Acta - General Subjects*. 1830(10), pp. 4719–4733. doi: 10.1016/j.bbagen.2013.06.006.

Miyata, S. *et al.* (2012) 'Persistent cortical plasticity by upregulation of chondroitin 6-sulfation', *Nature Neuroscience*. Nature Publishing Group, 15(3), pp. 414–422. doi: 10.1038/nn.3023.

Moon, L. D. F., Asher, R. A. and Fawcett, J. W. (2003) 'Limited growth of severed CNS axons after treatment of adult rat brain with hyaluronidase', *Journal of Neuroscience Research*, 71(1), pp. 23–37. doi: 10.1002/jnr.10449.

Morawski, M. *et al.* (2004) 'Perineuronal nets potentially protect against oxidative stress', *Experimental Neurology*, 188(2), pp. 309–315. doi: 10.1016/j.expneurol.2004.04.017.

Morellini, F. *et al.* (2010) 'Improved reversal learning and working memory and enhanced reactivity to novelty in mice with enhanced GABAergic innervation in the dentate gyrus', *Cerebral Cortex*, 20(11), pp. 2712–2727. doi: 10.1093/cercor/bhq017.

Oohira, A. *et al.* (2004) 'Neuroglycan C, a brain-specific part-time proteoglycan, with a particular multidomain structure', *Glycoconjugate Journal*, 21(1–2), pp. 53–57. doi: 10.1023/B:GLYC.0000043748.90896.83.

Pizzorusso, T. (2002) ‘Reactivation of Ocular Dominance Plasticity in the Adult Visual Cortex’, *Science*, 298(5596), pp. 1248–1251. doi: 10.1126/science.1072699.
Popp, S. *et al.* (2003) ‘Localization of aggrecan and versican in the developing rat central nervous system’, *Developmental Dynamics*, 227(1), pp. 143–149. doi: 10.1002/dvdy.10282.
Rauch, U. *et al.* (2004) ‘Cartilage link protein interacts with neurocan, which shows hyaluronan binding characteristics different from CD44 and TSG-6’, *Matrix Biology*, 22(8), pp. 629–639. doi: 10.1016/j.matbio.2003.11.007.
Romberg, C. *et al.* (2013) ‘Depletion of perineuronal nets enhances recognition memory and long-term depression in the perirhinal cortex’, *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 33(16), pp. 7057–7065.
doi: 10.1523/JNEUROSCI.6267-11.2013.
Rowlands, D. *et al.* (2018) ‘Aggrecan Directs Extracellular Matrix-Mediated Neuronal Plasticity’, *The Journal of Neuroscience*, 38(47), pp. 10102–10113.
doi: 10.1523/JNEUROSCI.1122-18.2018.
Sale, A. *et al.* (2007) ‘Environmental enrichment in adulthood promotes amblyopia recovery through a reduction of intracortical inhibition’, *Nature Neuroscience*, 10(6), pp. 679–681.
doi: 10.1038/nn1899.
Schwarting, G. A. *et al.* (2004) ‘Semaphorin 3A-mediated axon guidance regulates convergence and targeting of P2 odorant receptor axons’, *European Journal of Neuroscience*, 19(7), pp. 1800–1810. doi: 10.1111/j.1460-9568.2004.03304.x.
Schwarting, G. A. *et al.* (2018) ‘Semaphorin 3A Is Required for Guidance of Olfactory Axons in Mice’, *The Journal of Neuroscience*, 20(20), pp. 7691–7697.
doi: 10.1523/jneurosci.20-20-07691.2000.
Seeger, G. *et al.* (1994) ‘Mapping of perineuronal nets in the rat brain stained by colloidal iron hydroxide histochemistry and lectin cytochemistry’, *Neuroscience*, 58(2), pp. 371–388.
doi: 10.1016/0306-4522(94)90044-2.
Shen, Y. *et al.* (2009) ‘RPTPσ s Is a Receptor for Chondroitin Sulfate Proteoglycan, an Inhibitor of Neural Regeneration’, 326(10), pp. 592–597. doi: 10.1126/science.1178310.
Silbert, J. E. and Sugumaran, G. (2002) ‘Biosynthesis of Chondroitin / Dermatan Sulfate’, *IUBMB life*, 54, pp. 177–186. doi: 10.1080/15216540290114450.
Smith-Thomas, L. C. *et al.* (1995) ‘Increased axon regeneration in astrocytes grown in the presence of proteoglycan synthesis inhibitors’, *Journal of Cell Science*, 108(3), pp. 1307–1315.
Sohal, V. S. *et al.* (2009) ‘Parvalbumin neurons and gamma rhythms enhance cortical circuit

performance', *Nature*. Nature Publishing Group, 459(7247), pp. 698–702. doi: 10.1038/nature07991.
Spatazza, J. *et al.* (2013) 'Choroid-Plexus-Derived Otx2 Homeoprotein Constrains Adult Cortical Plasticity', *Cell Reports*. The Authors, 3(6), pp. 1815–1823. doi: 10.1016/j.celrep.2013.05.014.
Spicer, A. P., Joo, A. and Bowling, R. A. (2003) 'A hyaluronan binding link protein gene family whose members are physically linked adjacent to chrondroitin sulfate proteoglycan core protein genes. The missing links', *Journal of Biological Chemistry*, 278(23), pp. 21083–21091. doi: 10.1074/jbc.M213100200.
Stern, R. *et al.* (2007) 'The many ways to cleave hyaluronan', *Biotechnology Advances*, 25(6), pp. 537–557. doi: 10.1016/j.biotechadv.2007.07.001.
Sugiyama, S. *et al.* (2008) 'Experience-Dependent Transfer of Otx2 Homeoprotein into the Visual Cortex Activates Postnatal Plasticity', *Cell*, 134(3), pp. 508–520. doi: 10.1016/j.cell.2008.05.054.
Sugiyama, S., Prochiantz, A. and Hensch, T. K. (2009) 'From brain formation to plasticity: Insights on Otx2 homeoprotein', *Development Growth and Differentiation*, 51(3), pp. 369–377. doi: 10.1111/j.1440-169X.2009.01093.x.
T. V. P. Bliss and G. L. Collingridge (1993) 'A synaptic model of memory: long-term potentiation in the hippocampus', *Nature*, 361(6407), pp. 31–39. doi: 10.1038/361031a0
Taniguchi, M. *et al.* (1997) 'Disruption of semaphorin III/D gene causes severe abnormality in peripheral nerve projection', *Neuron*, 19(3), pp. 519–530. doi: 10.1016/S0896-6273(00)80368-2.
Tucker, R. P. and Chiquet-Ehrismann, R. (2009) 'The regulation of tenascin expression by tissue microenvironments', *Biochimica et Biophysica Acta - Molecular Cell Research*. 1793(5), pp. 888–892. doi: 10.1016/j.bbamcr.2008.12.012.
Végh, M. J. *et al.* (2014) 'Reducing hippocampal extracellular matrix reverses early memory deficits in a mouse model of Alzheimer's disease.', *Acta neuropathologica communications*, 2(76), pp. 1-11. doi: 10.1186/s40478-014-0076-z.
Vo, T. *et al.* (2013) 'The chemorepulsive axon guidance protein semaphorin3A is a constituent of perineuronal nets in the adult rodent brain', *Molecular and Cellular Neuroscience*. 56, pp. 186–200. doi: 10.1016/j.mcn.2013.04.009.
Wang, D. and Fawcett, J. (2012) 'The perineuronal net and the control of cns plasticity', *Cell and Tissue Research*, 349(1), pp. 147–160. doi: 10.1007/s00441-012-1375-y.
Watanabe, E. *et al.* (1995) 'Distribution of a Brain-specific Proteoglycan, Neurocan, and the

Corresponding mRNA During the Formation of Barrels in the Rat Somatosensory Cortex', *European Journal of Neuroscience*, 7(4), pp. 547–554.
doi: 10.1111/j.1460-9568.1995.tb00659.x
Weber, P. *et al.* (1999) 'Mice deficient for tenascin-R display alterations of the extracellular matrix and decreased axonal conduction velocities in the CNS.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 19(11), pp. 4245–4262.
doi: https://doi.org/10.1523/JNEUROSCI.19-11-04245.1999
Wegner, F., *et al.* (2003). 'Diffuse perineuronal nets and modified pyramidal cells immunoreactive for glutamate and the GABA(A) receptor alpha1 subunit form a unique entity in rat cerebral cortex.', *Experimental Neurology*, 184, pp. 705–714.
doi: 10.1016/S0014-4886(03)00313-3
Weigel, P. H., Hascall, V. C. and Tammi, M. (1997) 'Hyaluronan synthases', *Journal of Biological Chemistry*, 272(22), pp. 13997–14000. doi: 10.1074/jbc.272.22.13997.
Winters, B. D., Saksida, L. M. and Bussey, T. J. (2008) 'Object recognition memory: Neurobiological mechanisms of encoding, consolidation and retrieval', *Neuroscience and Biobehavioral Reviews*, 32(5), pp. 1055–1070. doi: 10.1016/j.neubiorev.2008.04.004.
Xiang, J. and Brown, M. W. (1998) 'Differential neural encoding of novelty, familiarity and recency in regions of the anterior temporal lobe', 37, pp. 657–676.
doi: https://doi.org.10.1016/S0028-3908(98)00030-6
Yamada, H. *et al.* (1994) 'Molecular cloning of brevican, a novel brain proteoglycan of the aggrecan/versican family', *Journal of Biological Chemistry*, 269(13), pp. 10119–10126.
doi: 10.1016/S1044-7431(03)00133-7
Yamagata, T. *et al.* (1968) 'Purification and properties of bacterial chondroitinases and chondrosulfatases.', *Journal of Biological Chemistry*, 243(7), pp. 1523–1535.
Yamaguchi, Y. (2000) 'Lecticans: organizers of the brain extracellular matrix', *CMLS, Cell. Mol. Life Sci*, 57, pp. 276–289. doi: 10.1007/PL00000690.
Yang, S. *et al.* (2015) 'Perineuronal net digestion with chondroitinase restores memory in mice with tau pathology', *Experimental Neurology*. 265, pp. 48–58.
doi: 10.1016/j.expneurol.2014.11.013.
Yang, S., Hilton, S., Alves, J. N., *et al.* (2017) 'Antibody recognizing 4-sulfated chondroitin sulfate proteoglycans restores memory in tauopathy-induced neurodegeneration', *Neurobiology of Aging*. 59, pp. 197–209. doi: 10.1016/j.neurobiolaging.2017.08.002.
Ye, Q. and Miao, Q. long (2013) 'Experience-dependent development of perineuronal nets and chondroitin sulfate proteoglycan receptors in mouse visual cortex', *Matrix Biology*.

International Society of Matrix Biology, 32(6), pp. 352–363.
doi: 10.1016/j.matbio.2013.04.001.
Yoshioka, N. *et al.* (2017) 'Abnormalities in perineuronal nets and behavior in mice lacking CSGalNAcT1, a key enzyme in chondroitin sulfate synthesis', *Molecular Brain*. Molecular Brain, 10(1), pp. 1–10. doi: 10.1186/s13041-017-0328-5.
Zhu, X. *et al.* (1996) 'Mapping visual recognition memory through expression of the immediate early gene c-fos', *NeuroReport*, 7, pp. 1871–1875.
doi: 10.1097/00001756-199607290-00037